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to the Assistant Commissioner for Patents:

Transmitted herewith for filing under 35 U.S.C. 111 and 37 CFR 1.53 is the Continuation-in-Part application of:

Susan L. Acton, Keith E. Robison and Frank Y. Hsieh

entitled: Angiotensin Converting Enzyme Homolog and Therapeutic and Diagnostic Uses Therefor

Enclosed are:

- (X) 106 pages of written description, claims and abstract.  
 (X) 13 sheets of drawings (Figures 1A-7).  
 ( ) an assignment of the invention to \_\_\_\_\_  
 ( ) executed declaration of the inventors.  
 ( ) a certified copy of a \_\_\_\_\_ application.  
 ( ) a verified statement to establish small entity status under 37 CFR 1.9 and 1.27.  
 (X) other: unexecuted Declaration for Patent Application.

## CLAIMS AS FILED

	# FILED	# EXTRA	Rate	FEE	Rate (Small Entity)	FEE
BASIC FEE			\$790		\$395	
TOTAL CLAIMS	-20 =	*	x \$22		x \$11	
INDEPENDENT CLAIMS	-3 =	*	x \$82		x \$41	
MULTIPLE DEPENDENT CLAIMS			\$270		\$135	
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Nancy Jonda

Date: September 30, 1998

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 Reg. No. (see attached)  
 Agent for Applicants

**Angiotensin Converting Enzyme Homolog and Therapeutic and  
Diagnostic Uses Therefor**

5     **Cross-Reference to Related Applications**

          This application is a continuation-in-part of application No. 08/989,299, entitled "*Angiotensin Converting Enzyme Homolog and Therapeutic and Diagnostic Uses Therefor*", filed December 11, 1997. This application is hereby incorporated by reference herein

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1.           **Background of The Invention**

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          Hypertension, or high blood pressure, is the most common disease affecting the heart and blood vessels. Statistics indicate that hypertension occurs in more than 50 million Americans. The prevalence of hypertension increases with age. Between 85 and 90% of cases are primary (i.e., essential) hypertension, i.e., a persistently elevated blood pressure that cannot be attributed to any particular organic cause. The remaining percentage of cases are secondary hypertension, i.e., elevated blood pressure having an identifiable underlying cause such as kidney disease and adrenal hypersecretion.

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          Hypertension is of considerable concern because of the harm it can do to the heart, brain, and kidneys if it remains uncontrolled. The heart is most commonly affected by high blood pressure. When blood pressure is high, the heart uses more energy in pumping against the increased resistance caused by the elevated arterial blood pressure. Because of the increased effort, the heart muscle thickens and the heart becomes enlarged and needs more oxygen. If it cannot meet the demands put on it, angina pectoris or even myocardial infarction may develop. Hypertension can result in numerous complications include left ventricular failure; atherosclerotic heart disease; retinal hemorrhages, exudates, papilledema, and vascular accidents; cerebrovascular insufficiency with or without stroke; and renal failure. An untreated hypertensive patient is at great risk of developing disabling or fatal left ventricular failure, myocardial infarction, cerebral hemorrhage or infarction, or renal failure at early age. Hypertension is the most important risk factor predisposing to stroke and is an important risk factor predisposing to coronary atherosclerosis.

          An abnormal blood pressure can also result from specific conditions or diseases, such as heart failure. Heart failure is a chronic or acute state that results when the heart is not capable of providing sufficient cardiac output to satisfy the metabolic needs of

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the body. Heart failure is commonly referred to as congestive heart failure (CHF), since symptoms of increased venous pressure (pulmonary congestion with left heart failure and peripheral edema with right heart failure) are often predominant. Symptoms and signs of CHF include fatigue, peripheral and pulmonary edema, and visceral congestion (e.g., dyspnea). These symptoms are produced by diminished blood flow to the various tissues of the body and by accumulation of excess blood in the various organs, that results from the heart being incapable of pumping out the blood. Heart failure can result from several underlying diseases, most commonly in industrialized nations from atherosclerotic coronary artery disease with myocardial infarction. Myocarditis, various cardiomyopathies, and valvular and congenital defects may also result in heart failure (Anderoli et al., Cecil: Essentials of Medicine, Third Edition, WB Saunders Company, 1993). A major problem in CHF is the inability of the failing left ventricle to maintain a normal blood pressure, thus resulting in increased pre- and afterload, and leading to progressive ventricular dilation with wall remodeling. Vasodilators which induce a reduction in pre- and afterload, i.e., reduction of the systemic vascular resistance and reduction of the peripheral vascular resistance, respectively, are currently used to treat CHF (Lionel H. Opie, Drugs for the Heart, Third Edition, WB Saunders Company, 1991).

One important system involved in regulating blood pressure is the renin-angiotensin-aldosterone system. In this system, renin, a proteolytic enzyme formed in the granules of the juxtaglomerular apparatus cells catalyzes the conversion of angiotensinogen (a plasma protein) into angiotensin I, a decapeptide. This inactive product is then cleaved by a converting enzyme, termed angiotensin converting enzyme (ACE) mainly in the lung, but also in the kidney and brain, to an octapeptide, angiotensin II, which is a potent vasoconstrictor and also stimulates the release of aldosterone. Aldosterone is an adrenal cortex hormone that promotes the retention of salt and water by the kidneys and thus increases plasma volume, resulting in an increase in blood pressure. Angiotensin II also stimulates the release of norepinephrine from neural cells which interacts with specific receptors on blood vessels, thereby resulting in an increase in calcium and vasoconstriction. Another mechanism by which angiotensin II induces vasoconstriction is by interacting with specific receptors on blood vessels, thereby resulting in an opening of calcium channels and an increase in calcium, resulting in vasoconstriction.

ACE, also referred to as peptidyl dipeptidase A (EC 3.4.15.1) and kininase II is a metallopeptidase, more particularly a zinc peptidase which hydrolyses angiotensin I and other biologically active polypeptides, such as kinins, e.g., bradykinin. Bradykinin is a vasodilator, which acts at least in part by inducing release of vasodilator prostaglandins, and

which is inactivated upon hydrolysis by ACE. Thus, ACE increases blood pressure at least in part by producing angiotensin II, a vasoconstrictor, and by inactivating bradykinin, a vasodilator. Bradykinin is also involved in other biological activities including mediation of pain and inflammatory reactions.

5           The role of ACE in regulating blood pressure is further demonstrated at least by the efficacy of ACE inhibitors in reducing hypertension and treating CHF in individuals. ACE inhibitors have major roles as vasodilators in hypertension and CHF and are among the most efficient drugs for treating these disorders (see, e.g., Opie et al., Angiotensin Converting Enzyme Inhibitors and Conventional Vasodilators, in Lionel H. Opie, Drugs for  
10   the Heart, Third Edition, WB Saunders Company, 1991, p106). Several clinical trials indicate that ACE inhibitors prolong survival in a broad spectrum of patients with myocardial infarction and heart failure, ranging from those who are asymptomatic with ventricular dysfunction to those who have symptomatic heart failure but are normotensive and hemodynamically stable. For example, one study demonstrated a 40% reduction in mortality  
15   at 6 months in patients with severe heart failure (The CONSENSUS Trial Study Group, N. Engl. J. Med. 316:1429 (1987); The CONSENSUS Trial Study Group, N. Engl. J. Med. 325:293 (1991)).

          ACE cleaves substrates other than angiotensin I and bradykinin. For example, ACE cleaves enkephalins, as well as heptapeptide and octapeptide enkephalin precursors.  
20   ACE also hydrolyzes the tridecapeptide neurotensin to a dipeptide and undecapeptide (Skidgel et al. In Neuropeptides and Their Peptidases, Ed. Turner AJ, Chichester, UK, Ellis-Horwood, (1987)). ACE can also cleave and thereby inactivate substance P (Skidgel et al., *supra*).

          Several ACE inhibitors are currently available on the market (e.g., Captopril,  
25   Enalapril, Fosinopril, Lisinopril, and Ramipril). However, ACE inhibitors in large doses can cause a variety of undesirable secondary effects including nephrotic syndrome, membranous glomerulonephritis, nephritis, and leukopenia, as well as angioedema.

          The isolation of novel nucleic acids encoding novel ACE proteins would be useful, e.g., in developing drugs which are capable of regulating the activity of ACE without  
30   having the negative secondary effects.

## **2.           Summary of the Invention**

          The present invention is based, at least in part, on the discovery of a novel  
35   gene encoding a novel human protein, having sequence homologies with known angiotensin



converting enzymes (ACEs). Thus, the newly identified proteins and nucleic acids described herein are referred to as "angiotensin converting enzyme-2" or "ACE-2". The human ACE-2 gene transcript is shown in Figure 1 (SEQ ID NO: 1) and includes 5' and 3' untranslated regions and a 2415 base pair open reading frame (SEQ ID NO: 3) encoding an 805 amino acid polypeptide having SEQ ID NO: 2. The mature protein, i.e., the full length protein without the signal sequence is comprised of about 787 amino acids. ACE-2 is expressed predominantly in kidney and testis. A nucleic acid comprising the cDNA encoding the full length human ACE-2 polypeptide has been deposited at the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD) on December 3, 1997 has been assigned ATCC Designation No. 209510.

An amino acid and nucleotide sequence analysis using the BLAST program (Altschul et al. (1990) J. Mol. Biol. 215:403) revealed that certain portions of the amino acid and nucleic acid sequences of the newly identified human ACE-2 protein and nucleic acid have a sequence similarity with certain regions of angiotensin converting enzymes. In particular, the amino acid sequence of the zinc binding domain, which is conserved in all ACE proteins identified to date and which is located in the catalytic site of the enzyme and necessary for catalytic activity, is also found in ACE-2. Amino acids which have been identified as either contacting the zinc atom and/or involved in the catalysis and are conserved among all ACE proteins, are present in ACE-2. Thus, ACE-2 is believed to share at least some of the biological activities of ACE proteins, in particular the peptidase activity. In fact, as shown herein (see Example 5.4), ACE-2 cleaves the C-terminal amino acid from angiotensin I to produce Ang (1-9). ACE-2 also comprises a transmembrane domain which is present in most ACE proteins and which is likely to mediate protein attachment to the cell membrane. Except for the presence of other small regions of homology between ACE-2 and known ACE proteins, the other portions of ACE-2 are significantly different from those of known ACE proteins.

In one aspect, the invention features isolated ACE-2 nucleic acid molecules. In one embodiment, the ACE-2 nucleic acid is from a vertebrate. In a preferred embodiment, the ACE-2 nucleic acid is from a mammal, e.g. a human. In an even more preferred embodiment, the nucleic acid has the nucleic acid sequence set forth in SEQ ID NO:1 and/or 3 or a portion thereof. The disclosed molecules can be non-coding, (e.g. a probe, antisense, or ribozyme molecules) or can encode a functional ACE-2 polypeptide (e.g. a polypeptide which specifically modulates biological activity, by acting as either an agonist or antagonist of at least one bioactivity of the human ACE-2 polypeptide). In one embodiment, the nucleic acid molecules can hybridize to the ACE-2 gene contained in ATCC designation No.

209510. In another embodiment, the nucleic acids of the present invention can hybridize to a vertebrate ACE-2 gene or to the complement of a vertebrate ACE-2 gene. In a further embodiment, the claimed nucleic acid can hybridize with a nucleic acid sequence shown in Figure 1 (SEQ ID NOs: 1 and 3) or complement thereof. In a preferred embodiment, the hybridization is conducted under mildly stringent or stringent conditions.

In further embodiments, the nucleic acid molecule is an ACE-2 nucleic acid that is at least about 70%, preferably about 80%, more preferably about 85%, and even more preferably at least about 90% or 95% homologous to the nucleic acid shown as SEQ ID NOs: 1 or 3 or to the complement of the nucleic acid shown as SEQ ID NOs: 1 or 3. In a further embodiment, the nucleic acid molecule is an ACE-2 nucleic acid that is at least about 70%, preferably at least about 80%, more preferably at least about 85% and even more preferably at least about 90% or 95% similar in sequence to the ACE-2 nucleic acid contained in ATCC designation No. 209510 or shown set forth in SEQ ID NOs: 1 and/or 3 or complement thereof.

The invention also provides probes and primers comprising substantially purified oligonucleotides, which correspond to a region of nucleotide sequence which hybridizes to at least about 6 at least about 10, and at least about 15, at least about 20, or preferably at least about 25 consecutive nucleotides of the sequence set forth as SEQ ID NO: 1 or complements of the sequence set forth as SEQ ID NO: 1 or naturally occurring mutants or allelic variants thereof. In preferred embodiments, the probe/primer further includes a label group attached thereto, which is capable of being detected.

For expression, the subject nucleic acids can be operably linked to a transcriptional regulatory sequence, e.g., at least one of a transcriptional promoter (e.g., for constitutive expression or inducible expression) or transcriptional enhancer sequence. Such regulatory sequences in conjunction with an ACE-2 nucleic acid molecule can provide a useful vector for gene expression. This invention also describes host cells transfected with said expression vector whether prokaryotic or eukaryotic and *in vitro* (e.g. cell culture) and *in vivo* (e.g. transgenic) methods for producing ACE-2 proteins by employing said expression vectors.

In another aspect, the invention features isolated ACE-2 polypeptides, preferably substantially pure preparations, e.g. of plasma purified or recombinantly produced polypeptides. The ACE-2 polypeptide can comprise a full length protein or can comprise smaller fragments corresponding to one or more particular motifs/domains, or fragments comprising at least about 5, 10, 25, 50, 75, 100, 125, 130, 135, 140 or 145 amino acids in length. In particularly preferred embodiments, the subject polypeptide has an ACE-2

bioactivity, for example, it is capable of interacting with and/or hydrolyzing a target peptide, such as angiotensin I or kinetensin.

In a preferred embodiment, the polypeptide is encoded by a nucleic acid which hybridizes with the nucleic acid sequence represented in SEQ ID NOs: 1 and 3. In a further preferred embodiment, the ACE-2 polypeptide is comprised of the amino acid sequence set forth in SEQ ID NO: 2. The subject ACE-2 protein also includes within its scope modified proteins, e.g. proteins which are resistant to post-translational modification, for example, due to mutations which alter modification sites (such as tyrosine, threonine, serine or asparagine residues), or which prevent glycosylation of the protein, or which prevent interaction of the protein with intracellular proteins involved in signal transduction.

The ACE-2 polypeptides of the present invention can be glycosylated, or conversely, by choice of the expression system or by modification of the protein sequence to preclude glycosylation, reduced carbohydrate analogs can also be provided. Glycosylated forms can be obtained based on derivatization with glycosaminoglycan chains. Also, ACE-2 polypeptides can be generated which lack an endogenous signal sequence (though this is typically cleaved off even if present in the pro-form of the protein).

In yet another preferred embodiment, the invention features a purified or recombinant polypeptide, which has the ability to modulate, e.g., mimic or antagonize, an activity of a wild-type ACE-2 protein, e.g., its ability to bind and/or hydrolyze angiotensin I or kinetensin or a peptide having a significant amino acid homology thereto. Preferably, the polypeptide comprises an amino acid sequence identical or homologous to a sequence designated in SEQ ID No: 2.

Another aspect of the invention features chimeric molecules (e.g., fusion proteins) comprising an ACE-2 protein. For instance, the ACE-2 protein can be provided as a recombinant fusion protein which includes a second polypeptide portion, e.g., a second polypeptide having an amino acid sequence unrelated (heterologous) to the ACE-2 polypeptide. A preferred ACE-2 fusion protein is an immunoglobulin-ACE-2 fusion protein, in which an immunoglobulin constant region is fused to an ACE-2 polypeptide.

Yet another aspect of the present invention concerns an immunogen comprising an ACE-2 polypeptide in an immunogenic preparation, the immunogen being capable of eliciting an immune response specific for an ACE-2 polypeptide; e.g. a humoral response, an antibody response and/or cellular response. In a preferred embodiment, the immunogen comprises an antigenic determinant, e.g. a unique determinant of a protein encoded by the nucleic acid set forth in SEQ ID NO: 1 or 3; or as set forth in SEQ ID NO:2.

A still further aspect of the present invention features antibodies and antibody preparations specifically reactive with an epitope of an ACE-2 protein.

The invention also features transgenic non-human animals which include (and preferably express) a heterologous form of an ACE-2 gene described herein, or which misexpress an endogenous ACE-2 gene (e.g., an animal in which expression of one or more of the subject ACE-2 proteins is disrupted). Such transgenic animals can serve as animal models for studying cellular and/or tissue disorders comprising mutated or mis-expressed ACE-2 alleles or for use in drug screening. Alternatively, such transgenic animals can be useful for expressing recombinant ACE-2 polypeptides.

The invention further features assays and kits for determining whether an individual's ACE-2 genes and/or proteins are defective or deficient (e.g in activity and/or level), and/or for determining the identity of ACE-2 alleles. In one embodiment, the method comprises the step of determining the level of ACE-2 protein, the level ACE-2 mRNA and/or the transcription rate of an ACE-2 gene. In another preferred embodiment, the method comprises detecting, in a tissue of the subject, the presence or absence of a genetic alteration, which is characterized by at least one of the following: a deletion of one or more nucleotides from a gene; an addition of one or more nucleotides to the gene; a substitution of one or more nucleotides of the gene; a gross chromosomal rearrangement of the gene; an alteration in the level of a messenger RNA transcript of the gene; the presence of a non-wild type splicing pattern of a messenger RNA transcript of the gene; and/or a non-wild type level of the ACE-2 protein.

For example, detecting a genetic alteration or the presence of a specific polymorphic region can include (i) providing a probe/primer comprised of an oligonucleotide which hybridizes to a sense or antisense sequence of an ACE-2 gene or naturally occurring mutants thereof, or 5' or 3' flanking sequences naturally associated with the ACE-2 gene; (ii) contacting the probe/primer with an appropriate nucleic acid containing sample; and (iii) detecting, by hybridization of the probe/primer to the nucleic acid, the presence or absence of the genetic alteration. Particularly preferred embodiments comprise: 1) sequencing at least a portion of an ACE-2 gene, 2) performing a single strand conformation polymorphism (SSCP) analysis to detect differences in electrophoretic mobility between mutant and wild-type nucleic acids; and 3) detecting or quantitating the level of an ACE-2 protein in an immunoassay using an antibody which is specifically immunoreactive with a wild-type or mutated ACE-2 protein.

Information obtained using the diagnostic assays described herein (alone or in conjunction with information on another genetic defect, which contributes to the same

disease) is useful for diagnosing or confirming that a symptomatic subject (e.g. a subject symptomatic for hypertension, hypotension, CHF, or a kinetensin-associated condition), has a genetic defect (e.g. in an ACE-2 gene or in a gene that regulates the expression of an ACE-2 gene), which causes or contributes to the particular disease or disorder. Alternatively, the information (alone or in conjunction with information on another genetic defect, which contributes to the same disease) can be used prognostically for predicting whether a non-symptomatic subject is likely to develop a disease or condition, which is caused by or contributed to by an abnormal ACE-2 activity or protein level (e.g. hypertension, hypotension, CHF, or a kinetensin-associated condition) in a subject. In particular, the assays permit to ascertain an individual's predilection to develop a condition associated with a mutation in ACE-2, where the mutation is a single nucleotide polymorphism (SNP). Based on the prognostic information, a doctor can recommend a regimen (e.g. diet or exercise) or therapeutic protocol useful for preventing or prolonging onset of the particular disease or condition in the individual.

In addition, knowledge of the particular alteration or alterations, resulting in defective or deficient ACE-2 genes or proteins in an individual, alone or in conjunction with information on other genetic defects contributing to the same disease (the genetic profile of the particular disease) allows customization of therapy for a particular disease to the individual's genetic profile, the goal of "pharmacogenomics". For example, an individual's ACE-2 genetic profile or the genetic profile of a disease or condition, to which ACE-2 genetic alterations cause or contribute, can enable a doctor: 1) to more effectively prescribe a drug that will address the molecular basis of the disease or condition; and 2) to better determine the appropriate dosage of a particular drug. For example, the expression level of ACE-2 proteins, alone or in conjunction with the expression level of other genes, known to contribute to the same disease, can be measured in many patients at various stages of the disease to generate a transcriptional or expression profile of the disease. Expression patterns of individual patients can then be compared to the expression profile of the disease to determine the appropriate drug and dose to administer to the patient.

The ability to target populations expected to show the highest clinical benefit, based on the ACE-2 or disease genetic profile, can enable: 1) the repositioning of marketed drugs with disappointing market results; 2) the rescue of drug candidates whose clinical development has been discontinued as a result of safety or efficacy limitations, which are patient subgroup-specific; and 3) an accelerated and less costly development for drug candidates and more optimal drug labeling (e.g. since the use of ACE-2 as a marker is useful for optimizing effective dose).

In another aspect, the invention provides methods for identifying a compound which modulates an ACE-2 activity, e.g. the interaction between an ACE-2 polypeptide and a target peptide, e.g., angiotensin I, a kinin, or kinetensin. In a preferred embodiment, the method includes the steps of (a) forming a reaction mixture including: (i) an ACE-2 polypeptide, (ii) an ACE-2 binding partner (e.g., a target peptide, such as angiotensin I or kinetensin), and (iii) a test compound; and (b) detecting interaction of the ACE-2 polypeptide and the ACE-2 binding protein. A statistically significant change (potentiation or inhibition) in the interaction of the ACE-2 polypeptide and ACE-2 binding protein in the presence of the test compound, relative to the interaction in the absence of the test compound, indicates a potential agonist (mimetic or potentiator) or antagonist (inhibitor) of ACE-2 bioactivity for the test compound. The reaction mixture can be a cell-free protein preparation, e.g., a reconstituted protein mixture or a cell lysate, or it can be a recombinant cell including a heterologous nucleic acid recombinantly expressing the ACE-2 binding partner.

In preferred embodiments, the step of detecting interaction of the ACE-2 and ACE-2 binding partner (e.g., angiotensin I or kinetensin) is a competitive binding assay.

In preferred embodiments, at least one of the ACE-2 polypeptide and the ACE-2 binding partner comprises a detectable label, and interaction of the ACE-2 and ACE-2 binding partner is quantified by detecting the label in the complex. The detectable label can be, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. In other embodiments, the complex is detected by an immunoassay.

The invention also provides a methods for identifying an ACE-2 therapeutic, comprising contacting in a reaction mixture an ACE-2 polypeptide, a target peptide or analog thereof or portion thereof, and a test compound, in conditions wherein, but for the presence of the test compound, the ACE-2 polypeptide cleaves one or more amino acids from the target peptide or analog thereof or portion thereof to produce an ACE-2 target peptide conversion product, and detecting the presence of at least one of the target peptide or analog thereof or portion thereof, the ACE-2 target peptide conversion product, and one or more amino acids. A preferred method for determining the presence and/or the amount of at least one of the target peptide or analog thereof or portion thereof, the ACE-2 target peptide conversion product, and one or more amino acids comprises obtaining a mass spectrum of the reaction mixture or of a part thereof.

Yet another exemplary embodiment provides an assay for screening test compounds to identify agents which modulate the amount of ACE-2 produced by a cell. In

one embodiment, the screening assay comprises contacting a cell transfected with a reporter gene operably linked to an ACE-2 promoter with a test compound and determining the level of expression of the reporter gene. The reporter gene can encode, e.g., a gene product that gives rise to a detectable signal such as: color, fluorescence, luminescence, cell viability, relief of a cell nutritional requirement, cell growth, and drug resistance. For example, the reporter gene can encode a gene product selected from the group consisting of chloramphenicol acetyl transferase, luciferase, beta-galactosidase and alkaline phosphatase.

Also within the scope of the invention are methods for treating diseases or disorders which are associated with an aberrant ACE-2 level or activity or which can benefit from modulation of the activity or level of ACE-2, in particular diseases or conditions which are improved by modulation of the level of one or more angiotensin I conversion products, e.g., by an increase or decrease in the production of Ang.(1-9), Ang.(1-5), and/or Ang.(1-8) (angiotensin II) or conditions that are improved by modulation of kinetensin or kinetensin or kinetensin (1-8) level. Thus, the invention provides methods for treating hypertension, CHF, inflammatory reactions, allergic reactions, and methods to reduce pain. The methods comprise administering, e.g., either locally or systemically to a subject, a pharmaceutically effective amount of a composition comprising an ACE-2 therapeutic. Depending on the condition, the therapeutic can be an ACE-2 agonist or an ACE-2 antagonist. For example, an ACE-2 antagonist therapeutic can be administered to a subject having hypertension or CHF. In another embodiment, an ACE agonist is administered locally to a subject to reduce the inflammation and pain resulting from an insect sting or bite, which was accompanied by an injection of bradykinin.

In a particular embodiment of the invention, an ACE-2 antagonist is administered to a subject alone or together with an ACE antagonist. Thus, a dual therapy comprising administering to a subject an antagonist of ACE-2 and an antagonist of ACE can be used to prevent the accumulation of angiotensin II, e.g., to thereby reduce the blood pressure of the subject and prevent the development or appearance of conditions related thereto.

The invention also provides methods for identifying other potential substrates of an ACE-2 polypeptide as well as the product of the enzymatic reaction. In a preferred embodiment, the method comprises contacting a preparation containing an ACE-2 polypeptide with a test compound, e.g., a peptide, for a time sufficient for the enzymatic reaction to occur, and subjecting the reaction mixture, or a portion thereof, to mass spectrometry. The comparison of the mass spectra of the test compound with that of the

reaction mixture after incubation to allow the enzymatic reaction to occur, will indicate whether the test compound was converted into a new compound, in which case the test compound is a substrate of the ACE-2 polypeptide.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

### 3. Brief Description of the Figures

Figure 1 (A-B) shows the nucleotide sequence of a full length cDNA encoding human ACE-2 including 5' and 3' untranslated regions and coding sequences (SEQ ID NO: 1) and the deduced amino acid sequence of the ACE-2 protein (SEQ ID NO 2). The signal sequence is underlined, and the zinc binding domain (ZBD) and transmembrane (TMD) domain are boxed.

Figure 2 (A-D) shows an alignment of the amino acid sequence of human ACE-2 having SEQ ID NO: 2 with human testicular ACE (hu-ACET; SEQ ID NO: 4; GenBank Accession No. P22966), murine testicular ACE (mu-ACET; SEQ ID NO: 5; GenBank Accession No. P22967), rabbit testicular ACE (rb-ACET; SEQ ID NO: 6; GenBank Accession No. P22968), human endothelial ACE (hu-ACE; SEQ ID NO: 7; GenBank Accession No. P12821; U.S. Patent No. 5,539,045 by Soubrier et al.; and described in Soubrier et al. (1988) Proc. Natl. Acad. Sci. USA 85:9386), murine endothelial ACE (mu-ACE; SEQ ID NO: 8; GenBank Accession No. P09470), rat endothelial ACE (rat-ACE; SEQ ID NO: 9; GenBank Accession No. P47820) and rabbit endothelial ACE (rb-ACE; SEQ ID NO: 10; GenBank Accession No. P12822). Stars indicate amino acids which are common to all sequences. Two dots indicate that all amino acids at that position are conserved and one dot indicates that two or more sequences share the amino acid at that position, but that at least one sequence has an amino acid that is not a conservative substitution at that position. The zinc binding domain (ZBD) and the transmembrane domain (TMD) are boxed.

Figure 3 (A-C) shows an amino acid alignment of the human ACE-2 protein having SEQ ID NO: 2 with human testicular ACE (HUM\_tACE; SEQ ID NO: 4; GenBank Accession No. P22966), human endothelial ACE (HUM\_ACE; SEQ ID NO: 7; GenBank Accession No. P12821, *Drosophila melanogaster* ACE (DROME\_ACE; SEQ ID NO: 11; GenBank Accession No. Q10714), and *C. elegans* ACE (CE\_ACE) SEQ ID NO: 12; GenBank Accession No. U56966). Stars indicate amino acids which are common to all sequences. Two dots indicate that all amino acids at that position are conserved and one dot indicates that two or more sequences share the amino acid at that position, but that at least



one sequence has an amino acid that is not a conservative substitution at that position. The zinc binding domain (ZBD) and the transmembrane domain (TMD) are boxed.

Figure 4 (panels A-D) show mass spectra of angiotensin I (panel A); a CHO cell lysate (negative control; panel B); a CHO cell lysate containing testicular ACE (positive control; panel C); and a CHO cell lysate containing ACE-2 (panel D).

Figure 5 shows the tandem mass spectrum of the product of angiotensin I conversion by ACE-2, i., the peptide at m/z 1183.53.

Figure 6 (panels A and B) shows a mass spectrum of a reaction mixture containing angiotensin I and Lisinopril and a CHO cell lysate containing testicular ACE (panel A) or a CHO cell lysate containing ACE-2 (panel B) incubated at 37 °C for 30 minutes.

Figure 7 shows at least some angiotensin I conversion products and enzymes catalyzing these reactions (E= endopeptidase, e.g., neprilysin; AP= aminopeptidase).

#### 4. Detailed Description of the Invention

##### 4.1. General

The invention is based at least in part on the discovery of a gene encoding a protein having regions which are significantly homologous to regions of known angiotensin converting enzymes (ACEs). Thus, the genes and proteins disclosed herein are referred to as Angiotensin Converting Enzyme 2 (ACE-2) genes and proteins. The sequence of the full length cDNA encoding ACE-2 was determined from a clone obtained from a cDNA library prepared from mRNA of a human heart of a subject who had congestive heart failure. The cDNA encoding the full length human ACE-2 protein and comprising 5' and 3' untranslated regions is 3396 nucleotides long and has the nucleotide sequence shown in Figure 1 and is set forth as SEQ ID NO: 1. The full length human ACE-2 protein is 805 amino acids long and has the amino acid sequence shown in Figure 1 and set forth in SEQ ID NO: 2. The coding portion (open reading frame) of SEQ ID NO: 1 is set forth as SEQ ID NO: 3 and corresponds to nucleotides 82 to 2496 of SEQ ID NO:1. The cDNA encoding the full length ACE-2 protein has been deposited at the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD) on December 3, 1997 has been assigned ATCC Designation No. 209510.

The protein comprises a signal peptide from amino acid 1 to amino acid 18, which is encoded by nucleotides 82 to 135 of SEQ ID NO: 1. Thus, the mature ACE-2 protein has 787 amino acids and has the amino acid sequence from amino acid 19 to amino acid 805 of SEQ ID NO: 2.

ACE-2 protein further comprises several functional domains. ACE-2 comprises a zinc binding domain (ZBD) from amino acid 374 to amino acid 378 of SEQ ID NO: 2, which is encoded by the nucleotide sequence from nucleotide 1201 to 1215 of SEQ ID NO: 1 and referred to herein as minimum zinc binding domain. It is in fact likely that at least some of the adjacent amino acids participate in binding zinc. This minimum zinc binding domain has the amino acid sequence HHEMGGH (SEQ ID NO: 14), and is identical to the zinc binding domain that is present in all ACE proteins (see below) which have been identified as being located in the catalytic site of the enzyme (Lattion et al. (1989) FEBS Letters 252:99). Since amino acids 372-381 of SEQ ID NO: 2 are conserved in all ACE proteins (see below), it is likely that amino acids 372, 373, 379, 380, and 381 of SEQ ID NO: 2 are involved in binding zinc. In addition, all the amino acids which have been reported as interacting with the zinc atom or involved in catalysis in ACE proteins are present in ACE-2. Thus, by comparison, His 374, 378 and Glu 402 are probably the amino acids coordinating the zinc atom and Glu 375 and His 417 are probably involved in catalysis. It is also believed that Glu 406 is involved in the catalytic activity of the enzyme.

ACE-2 also has a hydrophobic region in its C-terminal region, having the amino acid sequence from about amino acid 741 to about amino acid 765 of SEQ ID NO: 2 and is encoded by the nucleotide sequence from about nucleotide 2302 to about nucleotide 2376 of SEQ ID NO: 1. This hydrophobic region is likely to be a transmembrane domain, similar to that present in ACE proteins (see below).

A BLAST search (Altschul et al. (1990) J. Mol. Biol. 215:403) of the nucleic acid and the amino acid sequences of ACE-2 revealed that certain portions of the ACE-2 protein and cDNA have a significant homology to certain regions of previously identified angiotensin converting enzymes. Two forms of ACE proteins have been described previously: a larger form, referred to as endothelial or somatic ACE, since it is present in numerous somatic tissues, including vascular endothelium, renal tubular epithelium, ciliated gut epithelium, stimulated macrophages, areas of the brain and testis. The smaller form of ACE is referred to as the testicular form, since it is found essentially only in developing sperm cells in the testis.

The previously cloned mature endothelial human ACE protein consists of 1277 amino acid residues and is organized into two large homologous domains, each bearing a putative active site (Soubrier et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:9386). Each of these two domains contain short amino acid sequences identical to those located around critical residues of the active site of other metalloproteinases (thermmolysin, neutral endopeptidase, and collagenase) and therefore bears a putative active site. Zinc has been

reported as essential for the activity of ACE (Bunning and Riordan (1985) J. Inorg. Biochem. 24:183). Only one of these sites is probably involved in catalyzing angiotensin II, since only one Zn atom has been reported to be associated per ACE molecule. However, another study showed that both domains have activity, as shown by measuring the activity of each domain as a separate protein. The human ACE protein exist in a soluble and in a membrane bound form. Membrane attachment is likely to be mediated by the C-terminal hydrophobic sequence located near the carboxyterminus of the protein (Soubrier et al, *supra*), which is also present in ACE-2. The human testicular ACE contains 732 residues (including the signal peptide) contains only one of these two large repetitive domains of endothelial ACE, i.e., the carboxyterminal domain (WO 91/00354 and Ehlers et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:7741). This domain is located N-terminal of a 67 amino acids stretch and a serine-threonine rich region that is specific to the testicular form of ACE. Testicular ACE is encoded by the same gene as that encoding the larger ACE protein, but is encoded by a mRNA transcribed from a site located in the 12<sup>th</sup> intron of the ACE gene encoding the endothelial protein (Howard et al. (1990) Mol. Cell. Biol. 10:4294).

As shown in Figures 2 and 3, which show amino acid sequence alignments of ACE-2 with human, mouse, rabbit, rat, testicular and endothelial ACE proteins and *Drosophila* and *C. elegans* ACE proteins, certain portions of ACE-2 are homologous to certain regions of ACE proteins. In particular, the zinc binding domain is conserved in all ACE proteins. Thus, since the zinc binding domain is located in the catalytic site of the protein that is responsible for its peptidase activity, the function of ACE-2 is likely to be similar to that of other ACE proteins. In fact, as predicted by the homology between the amino acid sequences of ACE-2 and ACE, ACE-2 is capable of hydrolyzing angiotensin I and thereby cleaving off the last C-terminal amino acid (i.e., leucine) from angiotensin I. This 9 amino acid peptide ("Ang.(1-9)") can be further hydrolyzed by ACE into a 5 amino acid peptide containing the first five amino acids from angiotensin I (see Figure 7). It is highly probable that ACE-2 is capable of catalyzing the hydrolysis of other peptides, such as kinins and that it is involved in regulating blood pressure in a similar manner as endothelial ACE protein.

Another homology between ACE-2 and other ACE proteins is the presence of a transmembrane domain in the carboxy terminal portion of the proteins. Thus, ACE-2 can be in a membrane bound form. ACE proteins have also been found in a soluble form, which may result either from leakage of the protein from the surface or, from specific hydrolysis by

a protease, or the soluble form may be encoded by a differentially spliced mRNA. Accordingly, ACE-2 is believed to exist in a soluble form.

The amino acid sequence alignment indicates the existence of other regions of strong homology between ACE-2 and ACE proteins (see Figures 2 and 3). However, the overall similarity of ACE-2 with ACE proteins is relatively weak. In fact, the overall percent identity and similarity between human ACE-2 and the human testicular ACE protein (which is the ACE protein with which ACE-2 has the highest overall similarity) is about 42.9% and 62% respectively. At the nucleotide level, human ACE-2 and human testicular ACE have about 50.8% identity.

Northern blot hybridizations indicated that the mRNA encoding human ACE-2 is about 4 kb, which correlates with the size of the full length cDNA. ACE-2 mRNA is expressed predominantly in kidney and testis. Thus, the pattern of expression of ACE-2 is closer to that of endothelial ACE than to that of testicular ACE, which is expressed only in testis in the developing sperm cell.

Accordingly, the invention provides nucleic acids encoding ACE-2 proteins, fragments thereof and homologs or variants thereof. The invention also provides ACE-2 polypeptides, fragments thereof and homologs or variants thereof.

Based at least on the observation of sequence homologies between ACE-2 and angiotensin converting enzymes, as well as the fact that ACE-2 is capable of hydrolyzing angiotensin I into Ang.(1-9), the invention further provides methods and compositions for regulating arterial blood pressure, which can be used, e.g., for treating or preventing arterial hypertension or congestive heart failure. In addition, since ACEs have been shown to hydrolyze other peptides, e.g., kinins, such as bradykinin, the compositions of the invention can also be used as analgesics, or for treating inflammatory diseases or conditions. Based at least on the fact that ACE-2 is also homologous to the testicular ACE, methods and compositions of the invention could also be used to treat and prevent diseases or conditions relating to fertility. Furthermore, based on the observation, described herein, that ACE-2 catalyzes the hydrolysis of kinetensin, ACE-2 therapeutics can be used for treating and preventing diseases associated with excessive histamine release or abnormal blood vessel permeability. The invention further provides diagnostic and prognostic methods, e.g., methods for determining whether a subject is at risk of developing or has developed a disease associated with an aberrant ACE-2 activity, e.g., arterial hypertension and CHF. Such assays can, for example, consist of determining whether the subject has a genetic alteration in an ACE-2 gene. Also within the scope of the invention are methods for identifying ACE-2 therapeutics, i.e., compounds, which are either ACE-2 agonists or ACE-2 antagonists.

Other aspects of the invention are described below or will be apparent to those skilled in the art in light of the present disclosure.

5      4.2.      Definitions

For convenience, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided below.

The term "ACE-2 nucleic acid" refers to a nucleic acid encoding an ACE-2 protein, such as nucleic acids having SEQ ID NO: 1 or 3, fragments thereof, complement  
10 thereof, and derivatives thereof.

The terms "ACE-2 polypeptide" and "ACE-2 protein" are intended to encompass polypeptides comprising the amino acid sequence SEQ ID NO: 2, fragments thereof, and homologs thereof and include agonist and antagonist polypeptides.

The term "ACE-2 therapeutic" refers to various forms of ACE-2 polypeptides,  
15 as well as peptidomimetics, nucleic acids, or small molecules, which can modulate at least one activity of an ACE-2 polypeptide, e.g., interaction with and/or hydrolysis of a target peptide, by mimicking or potentiating (agonizing) or inhibiting (antagonizing) the effects of a naturally-occurring ACE-2 polypeptide. An ACE-2 therapeutic which mimics or potentiates the activity of a wild-type ACE-2 polypeptide is a "ACE-2 agonist". Conversely, an ACE-2  
20 therapeutic which inhibits the activity of a wild-type ACE-2 polypeptide is a "ACE-2 antagonist".

The term "agonist", as used herein, is meant to refer to an agent that mimics or upregulates (e.g. potentiates or supplements) an ACE-2 bioactivity. An ACE-2 agonist can be a wild-type ACE-2 protein or derivative thereof having at least one bioactivity of the  
25 wild-type ACE-2. An ACE-2 therapeutic can also be a compound that upregulates expression of an ACE-2 gene or which increases at least one bioactivity of an ACE-2 protein. An agonist can also be a compound which increases the interaction of an ACE-2 polypeptide with another molecule, e.g, a target peptide.

"Antagonist" as used herein is meant to refer to an agent that downregulates  
30 (e.g. suppresses or inhibits) at least one ACE-2 bioactivity. An ACE-2 antagonist can be a compound which inhibits or decreases the interaction between an ACE-2 protein and another molecule, e.g., a target peptide, such as angiotensin I or a kinin. Accordingly, a preferred antagonist is a compound which inhibits or decreases hydrolysis of a target peptide. An antagonist can also be a compound that downregulates expression of an ACE-2 gene or which  
35 reduces the amount of ACE-2 protein present. An ACE-2 antagonist can be a dominant

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negative form of an ACE-2 polypeptide, e.g., a form of an ACE-2 polypeptide which is capable of interacting with a target peptide, e.g., angiotensin I, but which is not capable of hydrolysing the target peptide. The ACE-2 antagonist can also be a nucleic acid encoding a dominant negative form of an ACE-2 polypeptide, an ACE-2 antisense nucleic acid, or a ribozyme capable of interacting specifically with an ACE-2 RNA. Yet other ACE-2 antagonists are molecules which bind to an ACE-2 polypeptide and inhibit its action. Such molecules include peptides, e.g., forms of ACE-2 target peptides which do not have biological activity, and which inhibit hydrolysis of target peptides by ACE-2 by competition with the target peptides. Thus, such peptides will bind the active site of ACE-2 and prevent it from interacting with target peptides, e.g., angiotensin I. Yet other ACE-2 antagonists include antibodies interacting specifically with an epitope of an ACE-2 molecule, such that binding interferes with hydrolysis. In yet another preferred embodiment, the ACE-2 antagonist is a small molecule, such as a molecule capable of inhibiting the interaction between an ACE-2 polypeptide and a target peptide and/or binding to the catalytic site of the enzyme. Alternatively, the small molecule can be antagonist by interacting with sites other than the catalytic site, and inhibit the catalytic activity of ACE-2 by, e.g., altering the tertiary or quaternary structure of the enzyme.

The term "ACE-2 substrate conversion product" or "ACE-2 target peptide conversion product" refers to a product, in particular a peptide, which result from the enzymatic cleavage of the ACE-2 substrate by ACE-2.

The term "kinetensin conversion product" refers to a product resulting from enzymatic cleavage of kinetensin by ACE-2, e.g., kinetensin (1-8) having SEQ ID NO: 24.

The term "angiotensin conversion product" refers to a peptide resulting from hydrolysis of angiotensin I. Examples of such peptides and the enzymes catalyzing their production are shown in Figure 7. Angiotensin conversion products include Ang.(1-9), Ang.(1-8), Ang.(1-7), Ang.(1-6), Ang.(1-5), Ang. III, and (des-Asp)AngI.

The term "angiotensin (1-9) or "Ang. (1-9)" refers to an angiotensin I peptide (DRVYIHPFHL; SEQ ID NO: 15) in which the C-terminal leucine is absent, i.e., a peptide having the amino acid sequence DRVYIHPFH (SEQ ID NO: 16). The term "angiotensin (1-8) or "Ang.(1-8)" refers to angiotensin II, i.e., a peptide having the amino acid sequence DRVYIHPF (SEQ ID NO: 17). The term "angiotensin (1-7)" or "Ang.(1-7)" refers to an angiotensin I peptide in which the last 3 C-terminal amino acids are absent, i.e., a peptide having the amino acid sequence DRVYIHP (SEQ ID NO: 18). The term "angiotensin (1-6) or "Ang.(1-6)" refers to an angiotensin I peptide in which the last 4 C-terminal amino acids

are absent, i.e., a peptide having the amino acid sequence DRVYIH (SEQ ID NO: 19). The term "angiotensin (1-5)" or "Ang.(1-5)" refers to an angiotensin I peptide in which the last 5 C-terminal amino acids are absent, i.e., a peptide having the amino acid sequence DRVYI (SEQ ID NO: 20). The term "(des-Asp)Ang. I" refers to an angiotensin I peptide lacking the N-terminal amino acid, i.e., a peptide having the amino acid sequence RVYIHPFHL (SEQ ID NO: 21). The term "angiotensin III" or "Ang. III" refers to an angiotensin I peptide lacking the C-terminal amino acid and the last two N-terminal amino acids, i.e., a peptide having the amino acid sequence RVYIHPF (SEQ ID NO: 22).

The term "allele", which is used interchangeably herein with "allelic variant" refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene. Alleles of a specific gene can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions, and insertions of nucleotides. An allele of a gene can also be a form of a gene containing a mutation.

The term "allelic variant of a polymorphic region of an ACE-2 gene" refers to a region of an ACE-2 gene having one of several nucleotide sequences found in that region of the gene in other individuals.

The term "analog of a target peptide of an ACE-2 polypeptide" refers to a compound which has sufficient structural similarities with the target peptide, that it shares a biological activity of the target peptide, e.g., binding of the target peptide to an ACE-2 polypeptide or the ability to be hydrolyzed by an ACE-2 polypeptide. An analog can be, e.g., a peptidomimetic or any modified peptide.

"Biological activity" or "bioactivity" or "activity" or "biological function", which are used interchangeably, for the purposes herein means an effector or antigenic function that is directly or indirectly performed by an ACE-2 polypeptide (whether in its native or denatured conformation), or by any subsequence thereof. Biological activities include binding to a target peptide, e.g., angiotensin I, a kinin, or kinetensin; binding to other proteins or molecules; the capability to catalyze hydrolysis of a target peptide (function as a carboxypeptidase or endopeptidase), e.g., angiotensin I, a kinin, or kinetensin; and interaction with a metal ion, e.g.,  $Zn^{2+}$ ,  $Co^{2+}$ , and  $Mn^{2+}$ . An ACE-2 bioactivity can be modulated by affecting directly an ACE-2 polypeptide. Alternatively, an ACE-2 bioactivity can be

modulated by modulating the level of an ACE-2 polypeptide, such as by modulating expression of an ACE-2 gene.

As used herein the term "bioactive fragment of an ACE-2 polypeptide" refers to a fragment of a full-length ACE-2 polypeptide, wherein the fragment specifically mimics or antagonizes the activity of a wild-type ACE-2 polypeptide. The bioactive fragment preferably is a fragment capable of interacting with a target peptide, such as angiotensin I or kinetensin.

The term "an aberrant activity", as applied to an activity of a polypeptide such as ACE-2, refers to an activity which differs from the activity of the wild-type or native polypeptide or which differs from the activity of the polypeptide in a healthy subject. An activity of a polypeptide can be aberrant because it is stronger than the activity of its native counterpart. Alternatively, an activity can be aberrant because it is weaker or absent relative to the activity of its native counterpart. An aberrant activity can also be a change in an activity. For example an aberrant polypeptide can interact with a different target peptide. A cell can have an aberrant ACE-2 activity due to overexpression or underexpression of the gene encoding ACE-2.

The term "abnormal blood pressure" refers to hypertension and hypotension.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A "chimeric polypeptide" or "fusion polypeptide" is a fusion of a first amino acid sequence encoding one of the subject ACE-2 polypeptides with a second amino acid sequence defining a domain (e.g. polypeptide portion) foreign to and not substantially homologous with any domain of an ACE-2 polypeptide. A chimeric polypeptide may present a foreign domain which is found (albeit in a different polypeptide) in an organism which also expresses the first polypeptide, or it may be an "interspecies", "intergenic", etc. fusion of polypeptide structures expressed by different kinds of organisms. In general, a fusion polypeptide can be represented by the general formula X-ACE-2-Y, wherein ACE-2 represents a portion of the polypeptide which is derived from an ACE-2 polypeptide, and X and Y are independently absent or represent amino acid sequences which are not related to an ACE-2 sequence in an organism, including naturally occurring mutants.



The term "nucleotide sequence complementary to the nucleotide sequence set forth in SEQ ID NO: x" refers to the nucleotide sequence of the complementary strand of a nucleic acid strand having SEQ ID NO: x. The term "complementary strand" is used herein interchangeably with the term "complement". The complement of a nucleic acid strand can be the complement of a coding strand or the complement of a non-coding strand. When referring to double stranded nucleic acids, the complement of a nucleic acid having SEQ ID NO: x refers to the complementary strand of the strand having SEQ ID NO: x or to any nucleic acid having the nucleotide sequence of the complementary strand of SEQ ID NO: x. When referring to a single stranded nucleic acid having the nucleotide sequence SEQ ID NO: x, the complement of this nucleic acid is a nucleic acid having a nucleotide sequence which is complementary to that of SEQ ID NO: x. The nucleotide sequences and complementary sequences thereof are always given in the 5' to 3' direction.

A "delivery complex" shall mean a targeting means (e.g. a molecule that results in higher affinity binding of a gene, protein, polypeptide or peptide to a target cell surface and/or increased cellular or nuclear uptake by a target cell). Examples of targeting means include: sterols (e.g. cholesterol), lipids (e.g. a cationic lipid, virosome or liposome), viruses (e.g. adenovirus, adeno-associated virus, and retrovirus) or target cell specific binding agents (e.g. ligands recognized by target cell specific receptors). Preferred complexes are sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene, protein, polypeptide or peptide is released in a functional form.

As is well known, genes may exist in single or multiple copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which all still code for polypeptides having substantially the same activity. The term "DNA sequence encoding an ACE-2 polypeptide" may thus refer to one or more genes within a particular individual. Moreover, certain differences in nucleotide sequences may exist between individual organisms, which are called alleles. Such allelic differences may or may not result in differences in amino acid sequence of the encoded polypeptide yet still encode a polypeptide with the same biological activity.

A disease, disorder or condition "associated with" or "characterized by" an aberrant ACE-2 activity refers to a disease, disorder or condition in a subject which is caused by or contributed to by an aberrant ACE-2 activity.

"Histamine-associated condition" refers to a condition, disease or disorder caused by or contributed to by excessive release of histamine, e.g., conditions resulting from

excessive endothelial cell contraction, leakage of plasma into the tissues, and/or vasodilation. Examples of such conditions include topical and systemic allergies, excema, asthma, and anaphylactic shock

5 "Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are identical at that position. A degree of homology or similarity or identity between nucleic acid sequences is a function of the number of identical or matching  
10 nucleotides at positions shared by the nucleic acid sequences. A degree of identity of amino acid sequences is a function of the number of identical amino acids at positions shared by the amino acid sequences. A degree of homology or similarity of amino acid sequences is a function of the number of amino acids, i.e. structurally related, at positions shared by the amino acid sequences. An "unrelated" or "non-homologous" sequence shares less than 40 %  
15 identity, though preferably less than 25 % identity, with one of the ACE-2 sequences of the present invention.

The term "interact" as used herein is meant to include detectable relationships or association (e.g. biochemical interactions) between molecules, such as interaction between protein-protein, protein-nucleic acid, nucleic acid-nucleic acid, and protein-small molecule or  
20 nucleic acid-small molecule in nature.

The term "isolated" as used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. For example, an isolated nucleic acid encoding one of the subject ACE-2 polypeptides preferably includes no more than 10  
25 kilobases (kb) of nucleic acid sequence which naturally immediately flanks the ACE-2 gene in genomic DNA, more preferably no more than 5kb of such naturally occurring flanking sequences, and most preferably less than 1.5kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant  
30 DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides.

"Kinetensin (1-8)" refers to kinetensin (SEQ ID NO: 23) lacking the C-terminal leucine, i.e., IARRHPYF (SEQ ID NO: 24).

5 The term "kinetensin associated condition" refers to a condition resulting from an abnormal level of kinetensin or kinetensin conversion product in plasma or in tissues of a subject and includes histamine associated conditions.

The term "modulation" as used herein refers to both upregulation (i.e., activation or stimulation (e.g., by agonizing or potentiating)) and downregulation (i.e. inhibition or suppression (e.g., by antagonizing, decreasing or inhibiting)).

10 The term "mutated gene" refers to an allelic form of a gene, which is capable of altering the phenotype of a subject having the mutated gene relative to a subject which does not have the mutated gene. If a subject must be homozygous for this mutation to have an altered phenotype, the mutation is said to be recessive. If one copy of the mutated gene is sufficient to alter the genotype of the subject, the mutation is said to be dominant. If a subject has one copy of the mutated gene and has a phenotype that is intermediate between  
15 that of a homozygous and that of a heterozygous subject (for that gene) , the mutation is said to be co-dominant.

20 The "non-human animals" of the invention include mammals such as rodents, non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc. Preferred non-human animals are selected from the rodent family including rat and mouse, most preferably mouse, though transgenic amphibians, such as members of the *Xenopus* genus, and transgenic chickens can also provide important tools for understanding and identifying agents which can affect, for example, embryogenesis and tissue formation. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which the recombinant gene is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that one of the recombinant ACE-2 genes is present and/or  
25 expressed or disrupted in some tissues but not others.

30 As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

The term "polymorphism" refers to the coexistence of more than one form of a gene or portion (e.g., allelic variant) thereof. A portion of a gene of which there are at least two different forms, i.e., two different nucleotide sequences, is referred to as a "polymorphic

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region of a gene". A polymorphic region can be a single nucleotide, the identity of which differs in different alleles. A polymorphic region can also be several nucleotides long.

A "polymorphic gene" refers to a gene having at least one polymorphic region.

As used herein, the term "promoter" means a DNA sequence that regulates  
5 expression of a selected DNA sequence operably linked to the promoter, and which effects  
expression of the selected DNA sequence in cells. The term encompasses "tissue specific"  
promoters, i.e. promoters, which effect expression of the selected DNA sequence only in  
specific cells (e.g. cells of a specific tissue). The term also covers so-called "leaky"  
promoters, which regulate expression of a selected DNA primarily in one tissue, but cause  
10 expression in other tissues as well. The term also encompasses non-tissue specific promoters  
and promoters that constitutively express or that are inducible (i.e. expression levels can be  
controlled).

The terms "protein", "polypeptide" and "peptide" are used interchangeably  
herein when referring to a gene product.

15 The term "recombinant protein" refers to a polypeptide of the present  
invention which is produced by recombinant DNA techniques, wherein generally, DNA  
encoding an ACE-2 polypeptide is inserted into a suitable expression vector which is in turn  
used to transform a host cell to produce the heterologous protein. Moreover, the phrase  
"derived from", with respect to a recombinant ACE-2 gene, is meant to include within the  
20 meaning of "recombinant protein" those proteins having an amino acid sequence of a native  
ACE-2 polypeptide, or an amino acid sequence similar thereto which is generated by  
mutations including substitutions and deletions (including truncation) of a naturally occurring  
form of the polypeptide.

"Small molecule" as used herein, is meant to refer to a composition, which has  
25 a molecular weight of less than about 5 kD and most preferably less than about 4 kD. Small  
molecules can be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids  
or other organic (carbon containing) or inorganic molecules. Many pharmaceutical  
companies have extensive libraries of chemical and/or biological mixtures, often fungal,  
bacterial, or algal extracts, which can be screened with any of the assays of the invention to  
30 identify compounds that modulate an ACE-2 bioactivity.

As used herein, the term "specifically hybridizes" or "specifically detects"  
refers to the ability of a nucleic acid molecule of the invention to hybridize to at least  
approximately 6, 12, 20, 30, 50, 100, 150, 200, 300, 350, 400 or 425 consecutive nucleotides  
of a vertebrate, preferably an ACE-2 gene.

The term "target peptide" refers to a peptide which can be hydrolyzed by an ACE or ACE-2 protein. Target peptides include angiotensin I, kinins such as bradykinin, kinetensin, enkephalins, and neuropeptides such as substance P.

5 "Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of one of the ACE-2 genes is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended.  
10 It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring forms of ACE-2 polypeptide.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., via an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of an ACE-2 polypeptide or, in the case of anti-sense expression from the transferred gene, the expression of a naturally-occurring form of the ACE-2 polypeptide is disrupted.  
15

20 As used herein, the term "transgene" means a nucleic acid sequence (encoding, e.g., one of the ACE-2 polypeptides, or an antisense transcript thereto) which has been introduced into a cell. A transgene could be partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can also be present in a cell in the form of an episome. A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal  
25  
30 expression of a selected nucleic acid.

A "transgenic animal" refers to any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by  
35 introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as

by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of one of the ACE-2 polypeptide, e.g. either agonistic or antagonistic forms. However, transgenic animals in which the recombinant ACE-2 gene is silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs described below. Moreover, "transgenic animal" also includes those recombinant animals in which gene disruption of one or more ACE-2 genes is caused by human intervention, including both recombination and antisense techniques.

The term "treating" as used herein is intended to encompass curing as well as ameliorating at least one symptom of the condition or disease.

The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

The term "wild-type allele" refers to an allele of a gene which, when present in two copies in a subject results in a wild-type phenotype. There can be several different wild-type alleles of a specific gene, since certain nucleotide changes in a gene may not affect the phenotype of a subject having two copies of the gene with the nucleotide changes.

#### 4.3. Nucleic Acids of the Present Invention

The invention provides ACE-2 nucleic acids, homologs thereof, and portions thereof. Preferred nucleic acids have a sequence at least 70%, and more preferably 75% homologous and more preferably 80% and even more preferably at least 85% homologous with a nucleotide sequence of an ACE-2 gene, e.g., such as a sequence shown in one of SEQ

ID NOs: 1 or 3 or complement thereof or the ACE-2 nucleic acid having ATCC Designation No. 209510. Nucleic acids at least 90%, more preferably 95%, and most preferably at least about 98-99% homologous with a nucleic sequence represented in one of SEQ ID NOs: 1 or 3 or complement thereof are of course also within the scope of the invention. In preferred  
5 embodiments, the nucleic acid is mammalian and in particularly preferred embodiments, includes all or a portion of the nucleotide sequence corresponding to the coding region of one of SEQ ID NOs: 1 or 3.

The invention also pertains to isolated nucleic acids comprising a nucleotide sequence encoding ACE-2 polypeptides, variants and/or equivalents of such nucleic acids.  
10 The term equivalent is understood to include nucleotide sequences encoding functionally equivalent ACE-2 polypeptides or functionally equivalent peptides having an activity of an ACE-2 protein such as described herein. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitution, addition or deletion, such as allelic variants; and will, therefore, include sequences that differ from the nucleotide sequence  
15 of the ACE-2 gene shown in SEQ ID NOs: 1 or 3 due to the degeneracy of the genetic code.

Preferred nucleic acids are vertebrate ACE-2 nucleic acids. Particularly preferred vertebrate ACE-2 nucleic acids are mammalian. Regardless of species, particularly preferred ACE-2 nucleic acids encode polypeptides that are at least 70%, 80%, 90%, or 95% similar or identical to an amino acid sequence of a vertebrate ACE-2 protein. In one  
20 embodiment, the nucleic acid is a cDNA encoding a polypeptide having at least one bio-activity of the subject ACE-2 polypeptide. Preferably, the nucleic acid includes all or a portion of the nucleotide sequence corresponding to the nucleic acid of SEQ ID No 1 or 3.

Still other preferred nucleic acids of the present invention encode an ACE-2 polypeptide which is comprised of at least 2, 5, 10, 25, 50, 100, 150 or 200 amino acid  
25 residues. For example, such nucleic acids can comprise about 50, 60, 70, 80, 90, or 100 base pairs. Also within the scope of the invention are nucleic acid molecules for use as probes/primer or antisense molecules (i.e. noncoding nucleic acid molecules), which can comprise at least about 6, 12, 20, 30, 50, 60, 70, 80, 90 or 100 base pairs in length.

Another aspect of the invention provides a nucleic acid which hybridizes under  
30 stringent conditions to a nucleic acid represented by SEQ ID NOs: 1 or 3 or complement thereof or the nucleic acid having ATCC Designation No. 209510. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in Current Protocols in Molecular Biology, John Wiley &  
35 Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be

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selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. Both temperature and salt may be varied, or temperature of salt concentration may be held constant while the other variable is changed. In a preferred embodiment, an ACE-2 nucleic acid of the present invention will bind to one of SEQ ID NOS 1 or 3 or complement thereof under moderately stringent conditions, for example at about 2.0 x SSC and about 40°C. In a particularly preferred embodiment, an ACE-2 nucleic acid of the present invention will bind to one of SEQ ID NOs: 1 or 3 or complement thereof under high stringency conditions.

Nucleic acids having a sequence that differs from the nucleotide sequences shown in one of SEQ ID NOs: 1 or 3 or complement thereof due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having a biological activity of an ACE-2 polypeptide) but differ in sequence from the sequence shown in the sequence listing due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode histidine) may result in "silent" mutations which do not affect the amino acid sequence of an ACE-2 polypeptide. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject ACE-2 polypeptides will exist among mammals. One skilled in the art will appreciate that these variations in one or more nucleotides (e.g., up to about 3-5% of the nucleotides) of the nucleic acids encoding polypeptides having an activity of an ACE-2 polypeptide may exist among individuals of a given species due to natural allelic variation.

Nucleic acids of the invention can encode one or more of the following domains of an ACE-2 protein: the signal peptide, the extracellular domain comprising the zinc binding domain, the transmembrane domain, and the cytoplasmic domain. The amino acid sequences of these domains in human ACE-2 (SEQ ID NO: 2) and the position of the nucleotide sequence in SEQ ID NO: 1 encoding these domains are indicated in Table I:

**Table I**  
**Position of Domains in Human ACE-2**

<u>Domain</u>	<u>Nucleotides</u>	<u>Amino acids</u>
signal sequence	82-135	1-18
extracellular domain	136-2301	19-740



min. zinc binding domain	1201-1215	374-378
transmembrane domain	2302-2376	741-765
cytoplasmic domain	2377-2496	766-805

5                   The polynucleotide sequence of the present invention may encode for a mature form of the ACE-2, i.e., a form of ACE-2 which does not comprise the leader peptide, e.g., an ACE-2 protein which does not comprise about amino acids 1-18 of SEQ ID NO: 2. For example, a preferred nucleic acid of the invention comprises at least a portion of a nucleotide sequence encoding ACE-2, but does not include about nucleotides 82-135 of SEQ ID NO:

10   1. The mature form of an ACE-2 polypeptide can be a secreted ACE-2 polypeptide or a membrane bound ACE-2 polypeptide. In fact, ACE has been found in the form of a membrane enzyme at the surface of the vascular endothelial cells and renal epithelial cells. Alternatively, ACE has also been observed to be a secreted protein, and has been found, e.g., in plasma (see, e.g., Erdos et al. (1987) Lab. Invest. 56:345, Cardwell et al. (1976) Science

15   191:1050; and Ryan et al. (1976) Tissue Cell 8:125). Thus, nucleic acids encoding secreted as well as membrane bound forms of ACE-2 proteins are within the scope of the invention.

                  In the case of ACE proteins, it has been reported that a soluble form of the enzyme results from proteolytic cleavage by a specific enzyme termed "secretase" (Parvathy et al. (1997) Biochem. J. 327:37). Thus, it is possible that a similar mechanism of

20   solubilization of ACE-2 proteins occur. Accordingly, in cases in which the ACE-2 protein is desired as a purely membrane form as opposed to a soluble form, it may be preferable to change the nucleotide sequence of ACE-2 such that it does not encode a site recognizable and cleavable by a secretase.

                  A recombinant soluble form of ACE-2 can be produced, e.g, by deleting at

25   least a portion of the transmembrane domain which spans amino acids 741-765 of SEQ ID NO: 2, such that the protein is not capable to localize itself to a cell membrane. Thus, nucleic acids of the invention include those which encode at least a portion of an ACE-2 protein, but which lacks a portion from about nucleotide 2302 to about nucleotide 2376 of SEQ ID NO:

30   1. For example, a preferred nucleic acid encoding a soluble human ACE-2 protein comprises a nucleotide sequence from about nucleotide 136 to about nucleotide 2301 of SEQ ID NO:

1. Preferred soluble ACE-2 proteins comprise at least a portion of the extracellular domain of ACE-2 which corresponds to about amino acid 19 to about amino acid 740 of SEQ ID NO: 2 and is encoded by the nucleotide sequence from about nucleotide 136 to about nucleotide 2301 of SEQ ID NO: 1 of SEQ ID NO:1.

The polynucleotide sequence may also encode a leader sequence, e.g., the natural leader sequence or a heterologous leader sequence. Human ACE-2 has a leader sequence from amino acid 1 to amino acid 18 of SEQ ID NO: 2. Accordingly, the polynucleotide may encode the natural ACE-2 leader sequence. Alternatively, the nucleic acid can be engineered such that the natural leader sequence is deleted and a heterologous leader sequence inserted in its place. The term "leader sequence" is used interchangeably herein with the term "signal peptide". For example, the desired DNA sequence may be fused in the same reading frame to a DNA sequence which aids in expression and secretion of the polypeptide from the host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of the polypeptide from the cell. The protein having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the protein.

The polynucleotide of the present invention may also be fused in frame to a marker sequence, also referred to herein as "Tag sequence" encoding a "Tag peptide", which allows for marking and/or purification of the polypeptide of the present invention. In a preferred embodiment, the marker sequence is a hexahistidine tag, e.g., supplied by a PQE-9 vector. Numerous other Tag peptides are available commercially. Other frequently used Tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, the pFLAG system (International Biotechnologies, Inc.), the pEZZ-protein A system (Pharmacia, NJ), and a 16 amino acid portion of the Haemophilus *influenza* hemagglutinin protein. Furthermore, any polypeptide can be used as a Tag so long as a reagent, e.g., an antibody interacting specifically with the Tag polypeptide is available or can be prepared or identified.

In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant protein, can allow purification of the expressed fusion protein by affinity chromatography using a  $\text{Ni}^{2+}$  metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase to provide the purified protein (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable

joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. John Wiley & Sons: 1992).

Other preferred ACE-2 fusion proteins include ACE polypeptide is a ACE-2-immunoglobulin (ACE-2-Ig) polypeptide. The ACE-2-Ig polypeptide can comprise the entire extracellular domain of ACE-2, e.g, human ACE-2, or a variant thereof. For example, an ACE-2-Ig polypeptide can comprise an amino acid sequences from about amino acid 1 to about amino acid 740 of SEQ ID NO: 2. A nucleic acid encoding an ACE-2Ig fusion protein can comprise, e.g., about nucleotides 82 to 2301 of SEQ ID NO: 1 fused in frame to a nucleic acid encoding a constant Ig chain. ACE-2-Ig fusion proteins can be prepared as described e.g., in U.S. Patent No.5,434,131.

As indicated by the examples set out below, ACE-2 protein-encoding nucleic acids can be obtained from mRNA present in any of a number of eukaryotic cells, e.g., from cardiac tissue or kidney. It should also be possible to obtain nucleic acids encoding ACE-2 polypeptides of the present invention from genomic DNA from both adults and embryos. For example, a gene encoding an ACE-2 protein can be cloned from either a cDNA or a genomic library in accordance with protocols described herein, as well as those generally known to persons skilled in the art. cDNA encoding an ACE-2 protein can be obtained by isolating total mRNA from a cell, e.g., a vertebrate cell, a mammalian cell, or a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding an ACE-2 protein can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention. The nucleic acid of the invention can be DNA or RNA or analogs thereof. A preferred nucleic acid is a cDNA represented by a sequence selected from the group consisting of SEQ ID NOs: 1 or 3.

Preferred nucleic acids encode a vertebrate ACE-2 polypeptide comprising an amino acid sequence that is at least about 60% homologous, more preferably at least about 70% homologous and most preferably at least about 80% homologous with an amino acid sequence contained in SEQ ID No: 2. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with an amino acid sequence represented in SEQ ID No: 2 are also within the

scope of the invention. In one embodiment, the nucleic acid is a cDNA encoding a peptide having at least one activity of the subject vertebrate ACE-2 polypeptide. Preferably, the nucleic acid includes all or a portion of the nucleotide sequence corresponding to the coding region of SEQ ID NOs:1 and 3.

5 Preferred nucleic acids encode a bioactive fragment of a vertebrate ACE-2 polypeptide comprising an amino acid sequence at least about 60% homologous or identical, more preferably at least about 70% homologous or identical and most preferably at least about 80% homologous or identical with an amino acid sequence of SEQ ID No: 2. Nucleic acids which encode polypeptides which are at least about 90%, more preferably at least about 10 95%, and most preferably at least about 98-99% homologous or identical, with an amino acid sequence represented in SEQ ID No: 2 are also within the scope of the invention.

Bioactive fragments of ACE-2 polypeptides can be polypeptides having one or more of the following biological activities: the capability to catalyze an enzymatic reaction, e.g., the hydrolysis of a target peptide, such as the hydrolysis of an angiotensin I peptide into 15 Ang.(1-9) peptide, the hydrolysis of a kinin or derivative thereof, e.g., bradykinin or hydrolysis of kinetensin; binding to a metal ion, e.g., zinc, interacting with a substrate, e.g., angiotensin I, a kinin, or kinetensin. A bioactive fragment of an ACE-2 polypeptide can also be a polypeptide having an analgesic activity, an anti-inflammatory activity and/or an anti-allergenic activity, the capability to modulate cell growth, to interact with another molecule, 20 e.g., a target peptide or a receptor. Assays for determining whether an ACE-2 polypeptide has any of these or other biological activities are known in the art and are further described herein.

Nucleic acids encoding proteins having an ACE-2 activity include nucleic acids comprising a nucleotide sequence encoding a zinc binding domain, such as the zinc binding 25 domain of ACE-2 consisting of about amino acids 374-378 of SEQ ID NO: 2. Such a nucleic acid can be represented by the generic formula: X-(ZBD)-Y, wherein ZBD represents nucleotides 1201-1215 of SEQ ID NO: 1, and X and Y represent a certain number of nucleotides located 5' and 3' of the ZBD, respectively. For example, a nucleic acid of the invention can comprise nucleotides 1201-1215 of SEQ ID NO: 1 and X and Y selected from 30 any of 0, 10, 20, 30, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, or about 1000 nucleotides. Alternatively, ZBD can represent the nucleotide sequence from nucleotide 1192 to 1221 or 1195 to 1224 of SEQ ID NO: 1 or any sequence substantially similar thereto, or comprising one or more nucleotides at the 5' and/or 3' end. Another preferred ZBD is an extended ZBD, i.e., comprising nucleotides encoding amino acids which are located outside 35 of amino acids 371-380, but which are functionally active. For example, a preferred nucleic

acid comprises a nucleotide sequence from about nucleotide 1201 to about nucleotide 1331 of SEQ ID NO: 1 and encoding all the amino acids which either contact the zinc atom or which are involved in catalysis. These nucleic acids preferably encode a protein having a biological activity, e.g., the capability to catalyze the hydrolysis of a peptide. Alternatively such polypeptides are devoid of biological activity. Accordingly, the invention provides nucleic acids comprising nucleotide sequence from about nucleotide 1201 to about nucleotide 1331 of SEQ ID NO: 1, in which codons encoding residues 374, 375, 378, 402, 406, and/or 417 are mutated.

Nucleic acids encoding modified forms or mutant forms of ACE-2 also include those encoding ACE-2 proteins having mutated glycosylation sites, such that either the encoded ACE-2 protein is not glycosylated, partially glycosylated and/or has a modified glycosylation pattern. Seven potential N-linked glycosylation sites have been identified in human ACE-2 and these are located at amino acids 53, 90, 103, 322, 432, 546, and 690 in SEQ ID NO: 2. Glycosylation sites, N-glycosylation or O-glycosylation sites can also be added to the protein. Amino acid sequence motifs required for the attachment of a sugar unit are well known in the art.

Other preferred nucleic acids of the invention include nucleic acids encoding derivatives of ACE-2 polypeptides which lack one or more biological activities of ACE-2 polypeptides. For example, the invention provides derivatives of ACE-2 polypeptides having an anti-inflammatory activity but which are essentially incapable of hydrolyzing angiotensin I. Such nucleic acids can be obtained, e.g., by a first round of screening of libraries for the presence or absence of a first activity and a second round of screening for the presence or absence of another activity.

Also within the scope of the invention are nucleic acids encoding splice variants or nucleic acids representing transcripts synthesized from an alternative transcriptional initiation site, such as those whose transcription was initiated from a site in an intron., as is the case with the testicular ACE mRNAs. Such homologs can be cloned by hybridization or PCR, as further described herein.

In preferred embodiments, the ACE-2 nucleic acids can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup B. et al. (1996) *Bioorganic & Medicinal Chemistry* 4 (1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAS" refer to nucleic acid mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural

nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup B. et al. (1996) *supra*; Perry-O'Keefe et al. *PNAS* 93: 14670-675.

5 PNAs of ACE-2 can be used in therapeutic and diagnostic applications and are further described herein in section 4.3.2. Such modified nucleic acids can be used as antisense or antigene agents for sequence-specific modulation of gene expression or in the analysis of single base pair mutations in a gene by, e.g., PNA directed PCR clamping or as probes or primers for DNA sequence and hybridization (Hyrup B. et al (1996) *supra*; Perry-O'Keefe  
10 *supra*).

PNAs of ACE-2 can further be modified, e.g., to enhance their stability or cellular uptake, e.g., by attaching lipophilic or other helper groups to the ACE-2 PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. ACE-2 PNAs can also be linked to DNA as described, e.g., in  
15 Hyrup B. (1996) *supra* and Finn P.J. et al. (1996) *Nucleic Acids Research* 24 (17): 3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, e.g., 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag, M. et al. (1989) *Nucleic Acid Res.* 17: 5973-88). PNA  
20 monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5'PNA segment and a 3' DNA segment (Finn P.J. et al. (1996) *supra*). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K.H. et al. (1975) *Bioorganic Med Chem. Lett.* 5: 1119-11124).

In other embodiments, ACE-2 nucleic acids may include other appended  
25 groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents that facilitate transport across the cell membrane as described in section 4.3.2. herein.

#### 4.3.1. Probes and Primers

The nucleotide sequences determined from the cloning of ACE-2 genes from  
30 mammalian organisms will further allow for the generation of probes and primers designed for use in identifying and/or cloning ACE-2 homologs in other cell types, e.g., from other tissues, as well as ACE-2 homologs from other mammalian organisms. For instance, the present invention also provides a probe/primer comprising a substantially purified oligonucleotide, which oligonucleotide comprises a region of nucleotide sequence that  
35 hybridizes under stringent conditions to at least approximately 12, preferably 25, more

preferably 40, 50 or 75 consecutive nucleotides of sense or anti-sense sequence selected from the group consisting of SEQ ID No: 1 or 3 or naturally occurring mutants thereof. For instance, primers based on the nucleic acid represented in SEQ ID NOs:1 or 3 can be used in PCR reactions to clone ACE-2 homologs.

5 Likewise, probes based on the subject ACE-2 sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins, for use, e.g., in prognostic or diagnostic assays (further described below). In preferred embodiments, the probe further comprises a label group attached thereto and able to be detected, e.g., the label group is selected from amongst radioisotopes, fluorescent compounds, enzymes, and enzyme  
10 co-factors.

Probes and primers can be prepared and modified as described in the other sections herein relating to nucleic acids.

#### 4.3.2 Antisense, Ribozyme and Triplex techniques

15 Another aspect of the invention relates to the use of the isolated nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or in situ generation of oligonucleotide molecules or their derivatives which specifically hybridize (e.g., bind) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding one or more of the subject ACE-2 proteins so as to inhibit expression of that protein, e.g., by  
20 inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

25 An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes an ACE-2 protein. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing  
30 with the mRNA and/or genomic sequences of an ACE-2 gene. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, e.g., exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and  
35 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense

therapy have been reviewed, for example, by Van der Krol et al. (1988) BioTechniques 6:958-976; and Stein et al. (1988) Cancer Res 48:2659-2668. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between the -10 and +10 regions of the ACE-2 nucleotide sequence of interest, are preferred.

5                   Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to ACE-2 mRNA. The antisense oligonucleotides will bind to the ACE-2 mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. In the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The  
10                   ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

15                   Oligonucleotides that are complementary to the 5' end of the mRNA, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well. (Wagner, R. 1994. Nature 372:333). Therefore, oligonucleotides  
20                   complementary to either the 5' or 3' untranslated, non-coding regions of an ACE-2 gene could be used in an antisense approach to inhibit translation of endogenous ACE-2 mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could also be used in  
25                   accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region of ACE-2 mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably less than about 100 and more preferably less than about 50, 25, 17 or 10 nucleotides in length.

30                   Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally,  
35                   it is envisioned that results obtained using the antisense oligonucleotide are compared with

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those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

5           The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other  
10           appended groups such as peptides (e.g., for targeting host cell receptors), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. W088/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. W089/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., 1988, BioTechniques 6:958-976) or  
15           intercalating agents. (See, e.g., Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

          The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil,  
20           5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-  
25           methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil,  
30           (acp3)w, and 2,6-diaminopurine.

          The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

          The antisense oligonucleotide can also contain a neutral peptide-like backbone.  
35           Such molecules are termed peptide nucleic acid (PNA)-oligomers and are described, e.g., in

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Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93:14670 and in Eglom et al. (1993) Nature 365:566. One advantage of PNA oligomers is their capability to bind to complementary DNA essentially independently from the ionic strength of the medium due to the neutral backbone of the DNA. In yet another embodiment, the antisense oligonucleotide  
5 comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet a further embodiment, the antisense oligonucleotide is an  $\alpha$ -anomeric oligonucleotide. An  $\alpha$ -anomeric oligonucleotide forms specific double-stranded hybrids with  
10 complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods  
15 known in the art, e.g., by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass  
20 polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

While antisense nucleotides complementary to the ACE-2 coding region  
sequence can be used, those complementary to the transcribed untranslated region and to the region comprising the initiating methionine are most preferred.

The antisense molecules can be delivered to cells which express ACE-2 *in*  
25 *vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systematically.

However, it may be difficult to achieve intracellular concentrations of the  
30 antisense sufficient to suppress translation on endogenous mRNAs in certain instances. Therefore a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of  
35 sufficient amounts of single stranded RNAs that will form complementary base pairs with the

endogenous ACE-2 transcripts and thereby prevent translation of the ACE-2 mRNA. For example, a vector can be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al, 1982, Nature 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site. Alternatively, viral vectors can be used which selectively infect the desired tissue, in which case administration may be accomplished by another route (e.g., systematically).

Ribozyme molecules designed to catalytically cleave ACE-2 mRNA transcripts can also be used to prevent translation of ACE-2 mRNA and expression of ACE-2 (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225 and U.S. Patent No. 5,093,246). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy ACE-2 mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591. There are a number of potential hammerhead ribozyme cleavage sites within the nucleotide sequence of human ACE-2 cDNA (Fig. 1). Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the ACE-2 mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been extensively described

by Thomas Cech and collaborators (Zaug, et al., 1984, Science, 224:574-578; Zaug and Cech, 1986, Science, 231:470-475; Zaug, et al., 1986, Nature, 324:429-433; published International patent application No. WO88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes  
5 to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in an ACE-2 gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g., for improved stability, targeting, etc.) and should be delivered to cells  
10 which express the ACE-2 gene *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous ACE-2 messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

15 Endogenous ACE-2 gene expression can also be reduced by inactivating or "knocking out" the ACE-2 gene or its promoter using targeted homologous recombination. (E.g., see Smithies et al., 1985, Nature 317:230-234; Thomas & Capecchi, 1987, Cell 51:503-512; Thompson et al., 1989 Cell 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional ACE-2 (or a completely unrelated  
20 DNA sequence) flanked by DNA homologous to the endogenous ACE-2 gene (either the coding regions or regulatory regions of the ACE-2 gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express ACE-2 *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the ACE-2 gene. Such approaches are particularly suited in the agricultural  
25 field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive ACE-2 (e.g., see Thomas & Capecchi 1987 and Thompson 1989, *supra*). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

30 Alternatively, endogenous ACE-2 gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the ACE-2 gene (i.e., the ACE-2 promoter and/or enhancers) to form triple helical structures that prevent transcription of the ACE-2 gene in target cells in the body. (See generally, Helene, C. 1991, Anticancer Drug Des., 6(6):569-84; Helene, C., et al., 1992, Ann. N.Y. Acad. Sci., 660:27-  
35 36; and Maher, L.J., 1992, Bioassays 14(12):807-15).

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription are preferably single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides should promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

Antisense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Moreover, various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

#### 4.3.3. Vectors Encoding ACE-2 Proteins and ACE-2 Expressing Cells

The invention further provides plasmids and vectors encoding an ACE-2 protein, which can be used to express an ACE-2 protein in a host cell. The host cell may be any prokaryotic or eukaryotic cell. Thus, a nucleotide sequence derived from the cloning of mammalian ACE-2 proteins, encoding all or a selected portion of the full-length protein, can be used to produce a recombinant form of an ACE-2 polypeptide via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial) cells, are standard procedures well known in the art.

Vectors that allow expression of a nucleic acid in a cell are referred to as expression vectors. Typically, expression vectors used for expressing an ACE-2 protein contain a nucleic acid encoding an ACE-2 polypeptide, operably linked to at least one transcriptional regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject ACE-2 proteins. Transcriptional regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). In one embodiment, the expression vector includes a recombinant gene encoding a peptide having an agonistic activity of a subject ACE-2 polypeptide, or alternatively, encoding a peptide which is an antagonistic form of an ACE-2 protein.

Suitable vectors for the expression of an ACE-2 polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al. (1983) in Experimental Manipulation of Gene Expression, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 *ori*, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, an ACE-2 polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of one of the ACE-2 genes represented in SEQ ID NOs:1 or 3.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see Molecular Cloning A Laboratory Manual, 2<sup>nd</sup> Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In some instances, it may be desirable to express the recombinant ACE-2 polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the  $\beta$ -gal containing pBlueBac III).

When it is desirable to express only a portion of an ACE-2 protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) J. Bacteriol. 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) PNAS 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing ACE-2 derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Moreover, the gene constructs of the present invention can also be used as part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of one of the subject ACE-2 proteins. Thus, another aspect of the invention features expression vectors for *in vivo* or *in vitro* transfection and expression of an ACE-2

polypeptide in particular cell types so as to reconstitute the function of, or alternatively, abrogate the function of ACE-2 in a tissue. This could be desirable, for example, when the naturally-occurring form of the protein is misexpressed or the natural protein is mutated and less active.

5 In addition to viral transfer methods, non-viral methods can also be employed to cause expression of a subject ACE-2 polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral targeting means of the present invention rely on endocytic pathways for the uptake of the subject ACE-  
10 2 polypeptide gene by the targeted cell. Exemplary targeting means of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In other embodiments transgenic animals, described in more detail below could be used to produce recombinant proteins.

15 4.4. Polypeptides of the Present Invention

The present invention makes available isolated ACE-2 polypeptides which are isolated from, or otherwise substantially free of other cellular proteins. The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure or purified preparations" are defined as encompassing  
20 preparations of ACE-2 polypeptides having less than about 20% (by dry weight) contaminating protein, and preferably having less than about 5% contaminating protein. Functional forms of the subject polypeptides can be prepared, for the first time, as purified preparations by using a cloned gene as described herein.

Preferred ACE-2 proteins of the invention have an amino acid sequence which  
25 is at least about 60%, 70%, 80%, 85%, 90%, or 95% identical or homologous to an amino acid sequence of SEQ ID NO: 2. Even more preferred ACE-2 proteins comprise an amino acid sequence which is at least about 97, 98, or 99% homologous or identical to an amino acid sequence of SEQ ID NO: 2. Such proteins can be recombinant proteins, and can be, e.g., produced *in vitro* from nucleic acids comprising a nucleotide sequence set forth in SEQ  
30 ID NO: 1 or 3, or homologs thereof. For example, recombinant polypeptides preferred by the present invention can be encoded by a nucleic acid, which is at least 85% homologous and more preferably 90% homologous and most preferably 95 % homologous with a nucleotide sequence set forth in SEQ ID NOs: 1 or 3. Polypeptides which are encoded by a nucleic acid that is at least about 98-99% homologous with the sequence of SEQ ID NOs: 1 or 3 are also  
35 within the scope of the invention.



In a preferred embodiment, an ACE-2 protein of the present invention is a mammalian ACE-2 protein. In a particularly preferred embodiment an ACE-2 protein is set forth as SEQ ID No: 2. In particularly preferred embodiment, an ACE-2 protein has an ACE-2 bioactivity. It will be understood that certain post-translational modifications, e.g., phosphorylation and the like, can increase the apparent molecular weight of the ACE-2 protein relative to the unmodified polypeptide chain.

ACE-2 polypeptides preferably are capable of functioning in one of either role of an agonist or antagonist of at least one biological activity of a wild-type ("authentic") ACE-2 protein of the appended sequence listing. The term "evolutionarily related to", with respect to amino acid sequences of ACE-2 proteins, refers to both polypeptides having amino acid sequences which have arisen naturally, and also to mutational variants of human ACE-2 polypeptides which are derived, for example, by combinatorial mutagenesis.

Full length proteins or fragments corresponding to one or more particular motifs and/or domains or to arbitrary sizes, for example, at least 5, 10, 25, 50, 75 and 100, amino acids in length are within the scope of the present invention.

For example, isolated ACE-2 polypeptides can be encoded by all or a portion of a nucleic acid sequence shown in any of SEQ ID NOs: 1 or 3. Isolated peptidyl portions of ACE-2 proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, an ACE-2 polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") ACE-2 protein.

An ACE-2 polypeptide can be a membrane bound form or a soluble form. A preferred soluble ACE-2 polypeptide is a polypeptide which does not contain the transmembrane domain located from about amino acid 741 to about amino acid 765 of SEQ ID NO: 2. It is likely that there are natural forms of ACE-2 which fail to contain this domain. Alternatively, such proteins can be created by genetic engineering by methods known in the art. Soluble ACE-2 proteins can comprise an amino acid sequence from about amino acid 19 to about amino acid 740 of SEQ ID NO: 2 or homologs thereof. Such proteins can further comprise the amino acid sequence from about amino acid 765 to about amino acid 805 of SEQ ID NO: 2. Alternatively, soluble ACE-2 proteins can comprise the signal sequence, i.e.,

amino acids 1-18 of SEQ ID NO: 2 or a heterologous signal sequence, which is necessary for obtaining secretion of the protein.

It has been reported membraneous ACE proteins can become detached from the membrane to become soluble forms of ACE as the result of a post-translational proteolytic processing event. A metalloprotease responsible for this effect has been isolated and referred to as "secretase" (see, e.g., Parvathy et al. (1997) *Biochem. J.* 327:37). Accordingly, it is likely that ACE-2 proteins are similarly rendered soluble. Thus, the invention also provides ACE-2 proteins in which the site of cleavage is modified, such that a secretase would not be able to hydrolyze ACE-2.

In general, polypeptides referred to herein as having an activity (e.g., are "bioactive") of an ACE-2 protein are defined as polypeptides which include an amino acid sequence encoded by all or a portion of the nucleic acid sequences shown in one of SEQ ID NOs: 1 or 3 and which mimic or antagonize all or a portion of the biological/biochemical activities of a naturally occurring ACE-2 protein. Examples of such biological activity include the ability to catalyze hydrolysis of angiotensin I into Ang.(1-9), the ability to catalyze hydrolysis of a kinin or derivative thereof; the ability to catalyze the hydrolysis of kinetensin; the ability to bind to a metal ion, e.g., zinc, the ability to interact with a substrate, e.g., angiotensin I or a kinin, the ability to compete with ACE for a target peptide, e.g, angiotensin I; the ability to function as an analgesic, the ability to function as an anti-inflammatory agent the ability to modulate release of histamine from mast cells, and the ability to modulate blood cell wall permeability. Other biological activities of the subject ACE-2 proteins are described herein or will be reasonably apparent to those skilled in the art. According to the present invention, a polypeptide has biological activity if it is a specific agonist or antagonist of a naturally-occurring form of an ACE-2 protein.

A preferred ACE-2 polypeptide having a biological activity is an ACE-2 polypeptide comprising a zinc binding domain, e.g, an amino acid sequence from amino acid 374 to amino acid 378 of SEQ ID NO: 2. Such a zinc binding domain is present in all ACE proteins identified to this date and has been identified as being located in the catalytic site of the enzyme (Lattion et al. (1989) *FEBS Letters* 252:99). All the amino acids which have been reported as interacting with the zinc atom or involved in catalysis in ACE proteins are present in ACE-2. Thus, by comparison, His374, 378 and Glu402 are probably the amino acids coordinating the zinc atom and Glu375 and His417 are probably involved in catalysis, as well as Glu 406. Thus, preferred ACE-2 polypeptides can be generally represented by the formula: X-(ZBD)-Y, wherein ZBD corresponds to amino acids 374 to 378, 371 to 380, or 372 to 381 and X and Y represent a certain number of amino acids located upstream and downstream of

ZBD, respectively. X and Y can be any number of amino acids, including 0, 1, 2, 5, 10, 20, 50, 100, 200, or 300. The ZBD can also be an extended ZBD, e.g., including the amino acid sequence from about amino acid 374 to about amino acid 420 of SEQ ID NO: 2 and containing all the amino acids likely to be involved in the active site of the enzyme. ZBD can also be a mutant ZBD, which is, e.g., incapable of binding a zinc atom, and thus incapable of catalyzing the hydrolysis of a peptide. For example, a mutant ZBD can be a ZBD in which His 374, Glu 375, His 378, Glu 402, Glu 406, and/or His 417 are replaced by another amino acid. Mutated ACE-2 proteins can be used, e.g., as dominant negative ACE-2 proteins which is capable of interacting with the target peptide, but fails to hydrolyze the target peptide and thus competes with the wild-type ACE-2 or with an ACE protein.

Assays for determining whether a compound, e.g., a protein, such as an ACE-2 protein or variant thereof, has one or more of the above biological activities are well known in the art. For example, assays for determining whether an ACE-2 protein, homolog, or fragment thereof is capable of catalyzing hydrolysis of a peptide can be performed as described in the Examples. Peptides for use as test substrates can be prepared according to methods known in the art or can be obtained commercially.

Other preferred proteins of the invention are those encoded by the nucleic acids set forth in the section pertaining to nucleic acids of the invention. In particular, the invention provides fusion proteins, e.g., ACE-2-immunoglobulin fusion proteins. Such fusion proteins can provide, e.g., enhanced stability and solubility of ACE-2 proteins and may thus be useful in therapy. Fusion proteins can also be used to produce an immunogenic fragment of an ACE-2 protein. For example, the VP6 capsid protein of rotavirus can be used as an immunologic carrier protein for portions of the ACE-2 polypeptide, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of a subject ACE-2 protein to which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising ACE-2 epitopes as part of the virion. It has been demonstrated with the use of immunogenic fusion proteins utilizing the Hepatitis B surface antigen fusion proteins that recombinant Hepatitis B virions can be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of an ACE-2 protein and the poliovirus capsid protein can be created to enhance immunogenicity of the set of polypeptide antigens (see, for example, EP Publication No: 0259149; and Evans et al. (1989) Nature 339:385; Huang et al. (1988) J. Virol. 62:3855; and Schlienger et al. (1992) J. Virol. 66:2).

The Multiple antigen peptide system for peptide-based immunization can also be utilized to generate an immunogen, wherein a desired portion of an ACE-2 polypeptide is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric branching lysine core (see, for example, Posnett et al. (1988) JBC 263:1719 and Nardelli et al. (1992) J. Immunol. 148:914). Antigenic determinants of ACE-2 proteins can also be expressed and presented by bacterial cells.

In addition to utilizing fusion proteins to enhance immunogenicity, it is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the ACE-2 polypeptides of the present invention. For example, ACE-2 polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the ACE-2 polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)).

The present invention further pertains to methods of producing the subject ACE-2 polypeptides. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. Suitable media for cell culture are well known in the art. The recombinant ACE-2 polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant ACE-2 polypeptide is a fusion protein containing a domain which facilitates its purification, such as GST fusion protein.

Moreover, it will be generally appreciated that, under certain circumstances, it may be advantageous to provide homologs of one of the subject ACE-2 polypeptides which function in a limited capacity as one of either an ACE-2 agonist (mimetic) or an ACE-2 antagonist, in order to promote or inhibit only a subset of the biological activities of the naturally-occurring form of the protein. Thus, specific biological effects can be elicited by treatment with a homolog of limited function, and with fewer side effects relative to treatment with agonists or antagonists which are directed to all of the biological activities of naturally occurring forms of ACE-2 proteins.

Homologs of each of the subject ACE-2 proteins can be generated by mutagenesis, such as by discrete point mutation(s), or by truncation. For instance, mutation can give rise to homologs which retain substantially the same, or merely a subset, of the biological activity of the ACE-2 polypeptide from which it was derived. Alternatively,

antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to an ACE-2 receptor.

5 The recombinant ACE-2 polypeptides of the present invention also include homologs of the wildtype ACE-2 proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter ubiquitination or other enzymatic targeting associated with the protein.

10 ACE-2 polypeptides may also be chemically modified to create ACE-2 derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of ACE-2 proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

15 Modification of the structure of the subject ACE-2 polypeptides can be for such purposes as enhancing therapeutic or prophylactic efficacy, stability (e.g., ex vivo shelf life and resistance to proteolytic degradation), or post-translational modifications (e.g., to alter phosphorylation pattern of protein). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, or to produce specific antagonists thereof, are considered functional equivalents of the ACE-2 polypeptides  
20 described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. The substitutional variant may be a substituted conserved amino acid or a substituted non-conserved amino acid.

For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine,  
25 or a similar replacement of an amino acid with a structurally related amino acid (i.e. isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. In similar fashion, the amino acid repertoire can be grouped as (1)  
30 acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan;  
35

(5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, Biochemistry, 2<sup>nd</sup> ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional ACE-2 homolog (e.g., functional in the sense that the resulting polypeptide mimics or antagonizes the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method for generating sets of combinatorial mutants of the subject ACE-2 proteins as well as truncation mutants, and is especially useful for identifying potential variant sequences (e.g., homologs). The purpose of screening such combinatorial libraries is to generate, for example, novel ACE-2 homologs which can act as either agonists or antagonist, or alternatively, possess novel activities all together. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein.

In one embodiment, the variegated library of ACE-2 variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential ACE-2 sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of ACE-2 sequences therein.

There are many ways by which such libraries of potential ACE-2 homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential ACE-2 sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3<sup>rd</sup> Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp 273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents NOS. 5,223,409, 5,198,346, and 5,096,815).

Likewise, a library of coding sequence fragments can be provided for an ACE-2 clone in order to generate a variegated population of ACE-2 fragments for screening and subsequent selection of bioactive fragments. A variety of techniques are known in the art for generating such libraries, including chemical synthesis. In one embodiment, a library of coding sequence fragments can be generated by (i) treating a double stranded PCR fragment of an ACE-2 coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule; (ii) denaturing the double stranded DNA; (iii) renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products; (iv) removing single stranded portions from reformed duplexes by treatment with S1 nuclease; and (v) ligating the resulting fragment library into an expression vector. By this exemplary method, an expression library can be derived which codes for N-terminal, C-terminal and internal fragments of various sizes.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of ACE-2 homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate ACE-2 sequences created by combinatorial mutagenesis techniques.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of  $10^{26}$  molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays. To overcome this problem, a new technique has been developed recently, recursive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, PNAS USA 89:7811-7815; Yourvan et al., 1992, Parallel Problem Solving from Nature, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, Protein Engineering 6(3):327-331).

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The invention also provides for reduction of the ACE-2 proteins to generate mimetics, e.g., peptide or non-peptide agents, such as small molecules, which are able to disrupt binding of an ACE-2 polypeptide of the present invention with a molecule, e.g. target peptide. Thus, such mutagenic techniques as described above are also useful to map the determinants of the ACE-2 proteins which participate in protein-protein interactions involved in, for example, binding of the subject ACE-2 polypeptide to a target peptide. To illustrate, the critical residues of a subject ACE-2 polypeptide which are involved in molecular recognition of its receptor can be determined and used to generate ACE-2 derived peptidomimetics or small molecules which competitively inhibit binding of the authentic ACE-2 protein with that moiety. By employing, for example, scanning mutagenesis to map the amino acid residues of the subject ACE-2 proteins which are involved in binding other proteins, peptidomimetic compounds can be generated which mimic those residues of the ACE-2 protein which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of an ACE-2 protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) J Med Chem 29:295; and Ewenson et al. in Peptides: Structure and Function (Proceedings of the 9<sup>th</sup> American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al. (1985) Tetrahedron Lett 26:647; and Sato et al. (1986) J Chem Soc Perkin Trans 1:1231), and b-aminoalcohols (Gordon et al. (1985) Biochem Biophys Res Commun 126:419; and Dann et al. (1986) Biochem Biophys Res Commun 134:71).

Also within the scope of the invention are kinetensin conversion products, such as ACE-2 kinetensin conversion products. A preferred conversion product is kinetensin (1-8) (SEQ ID NO: 24). Analogs of such conversion products are also within the scope of the invention and can be, e.g., peptidomimetics.

#### 4.5. Anti-ACE-2 Antibodies and Uses Therefor

Another aspect of the invention pertains to an antibody specifically reactive with a mammalian ACE-2 protein, e.g., a wild-type or mutated ACE-2 protein. For example,



by using immunogens derived from an ACE-2 protein, e.g., based on the cDNA sequences, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, Antibodies: A Laboratory Manual ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide (e.g., a mammalian ACE-2 polypeptide or an antigenic fragment which is capable of eliciting an antibody response, or a fusion protein as described above). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an ACE-2 protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies. In a preferred embodiment, the subject antibodies are immunospecific for antigenic determinants of an ACE-2 protein of a mammal, e.g., antigenic determinants of a protein set forth in SEQ ID No: 2 or closely related homologs (e.g., at least 90% homologous, and more preferably at least 94% homologous).

Following immunization of an animal with an antigenic preparation of an ACE-2 polypeptide, anti- ACE-2 antisera can be obtained and, if desired, polyclonal anti-ACE-2 antibodies isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) Nature, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with a mammalian ACE-2 polypeptide of the present invention and monoclonal antibodies isolated from a culture comprising such hybridoma cells. In one embodiment anti-human ACE-2 antibodies specifically react with the protein encoded by a nucleic acid having SEQ ID NO: 1.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with one of the subject mammalian ACE-2 polypeptides. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab)<sub>2</sub> fragments can be generated by treating antibody with pepsin. The resulting F(ab)<sub>2</sub> fragment

can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific, single-chain, and chimeric and humanized molecules having affinity for an ACE-2 protein conferred by at least one CDR region of the antibody. In preferred embodiments, the antibodies, the antibody further comprises a label attached thereto and able to be detected, (e.g., the label can be a radioisotope, fluorescent compound, enzyme or enzyme co-factor).

Anti-ACE-2 antibodies can be used, e.g., to monitor ACE-2 protein levels in an individual for determining, e.g., whether a subject has a disease or condition associated with an aberrant ACE-2 protein level, or allowing determination of the efficacy of a given treatment regimen for an individual afflicted with such a disorder. The level of ACE-2 polypeptides may be measured from cells in bodily fluid, such as in blood samples.

Another application of anti-ACE-2 antibodies of the present invention is in the immunological screening of cDNA libraries constructed in expression vectors such as  $\lambda$ gt11,  $\lambda$ gt18-23,  $\lambda$ ZAP, and  $\lambda$ ORF8. Messenger libraries of this type, having coding sequences inserted in the correct reading frame and orientation, can produce fusion proteins. For instance,  $\lambda$ gt11 will produce fusion proteins whose amino termini consist of  $\beta$ -galactosidase amino acid sequences and whose carboxy termini consist of a foreign polypeptide. Antigenic epitopes of an ACE-2 protein, e.g., other orthologs of a particular ACE-2 protein or other paralogs from the same species, can then be detected with antibodies, as, for example, reacting nitrocellulose filters lifted from infected plates with anti-ACE-2 antibodies. Positive phage detected by this assay can then be isolated from the infected plate. Thus, the presence of ACE-2 homologs can be detected and cloned from other animals, as can alternate isoforms (including splice variants) from humans.

Antibodies specifically binding to an ACE-2 conversion product are also within the scope of the invention. In an illustrative embodiment, the invention provides antibodies which specifically bind to kinetensin (1-8) and not to kinetensin (1-9).

#### 4.6. Transgenic animals

The invention further provides for transgenic animals, which can be used for a variety of purposes, e.g., to identify ACE-2 therapeutics. Transgenic animals of the invention include non-human animals containing a heterologous ACE-2 gene or fragment thereof under the control of an ACE-2 promoter or under the control of a heterologous promoter. Accordingly, the transgenic animals of the invention can be animals expressing a transgene encoding a wild-type ACE-2 protein or fragment thereof or variants thereof,

including mutants and polymorphic variants thereof. Such animals can be used, e.g., to determine the effect of a difference in amino acid sequence of an ACE-2 protein from the sequence set forth in SEQ ID NO: 2, such as a polymorphic difference. These animals can also be used to determine the effect of expression of an ACE-2 protein in a specific site or for identifying ACE-2 therapeutics or confirming their activity *in vivo*.

The transgenic animals can also be animals containing a transgene, such as reporter gene, under the control of an ACE-2 promoter or fragment thereof. These animals are useful, e.g., for identifying ACE-2 drugs that modulate production of ACE-2, such as by modulating ACE-2 gene expression. An ACE-2 gene promoter can be isolated, e.g., by screening of a genomic library with an ACE-2 cDNA fragment and characterized according to methods known in the art.

Yet other non-human animals within the scope of the invention include those in which the expression of the endogenous ACE-2 gene has been mutated or "knocked out". These animals could be useful to determine whether the absence of ACE-2 will result in a specific phenotype, in particular whether these mice have or are likely to develop a specific disease, such as high susceptibility to inflammatory reactions. These animals are also useful for determining the effect of a specific amino acid difference in an ACE-2 gene. In fact these knock out animals can be crossed with transgenic animals expressing, e.g., a mutated form of ACE-2, thus resulting in an animal which expresses only the mutated protein and not the wild-type ACE-2 protein.

Methods for obtaining transgenic and knockout non-human animals are well known in the art.

#### 4.7. Screening Assays for ACE-2 Therapeutics

The invention further provides screening methods for identifying ACE-2 therapeutics, e.g., for treating diseases or conditions caused by, or contributed to by an abnormal ACE-2 activity or which can benefit from a modulation of an ACE-2 activity or protein level, e.g., hypertension and CHF. An ACE-2 therapeutic can be any type of compound, including a protein, a peptide, peptidomimetic, small molecule, and nucleic acid. A nucleic acid can be, e.g., a gene, an antisense nucleic acid, a ribozyme, or a triplex molecule. An ACE-2 therapeutic of the invention can be an agonist or an antagonist. Preferred ACE-2 agonists include ACE-2 proteins or derivatives thereof which mimic at least one ACE-2 activity, e.g., the capability to catalyze hydrolysis of a target peptide or nucleic acids encoding such. Other preferred agonists include compounds which are capable of increasing the production of an ACE-2 protein in a cell, e.g., compounds capable of

upregulating the expression of an ACE-2 gene, and compounds which are capable of enhancing an ACE-2 activity and/or the interaction of an ACE-2 protein with another molecule, such as a target peptide. Preferred ACE-2 antagonists include ACE-2 proteins which are dominant negative proteins, which, e.g., are capable of binding to, but not to hydrolyze target peptides. Other preferred antagonists include compounds which decrease or inhibit the production of an ACE-2 protein in a cell and compounds which are capable of downregulating expression of an ACE-2 gene, and compounds which are capable of downregulating an ACE-2 activity and/or interaction of an ACE-2 protein with another molecule, such as a target peptide, e.g., angiotensin I, a kinin, or kinetensin. In another preferred embodiment, an ACE-2 antagonist is a modified form of a target peptide, which is capable of interacting with the catalytic site of an ACE-2 protein, but which does not have biological activity, e.g., which is not vasopressive.

The invention also provides screening methods for identifying ACE-2 therapeutics which are capable of binding to an ACE-2 protein, e.g., a wild-type ACE-2 protein or a mutated form of an ACE-2 protein, and thereby modulate the catalytic activity of the ACE-protein or degrades or causes the ACE-2 protein to be degraded. For example, such an ACE-2 therapeutic can be an antibody or derivative thereof which interacts specifically with an ACE-2 protein (either wild-type or mutated).

Thus, the invention provides screening methods for identifying ACE-2 agonist and antagonist compounds, comprising selecting compounds which are capable of interacting with an ACE-2 protein or with a molecule interacting with an ACE-2 protein such as a target peptide and/or compounds which are capable of modulating the interaction of an ACE-2 protein with another molecule, such as a target peptide. In general, a molecule which is capable of interacting with an ACE-2 protein is referred to herein as "ACE-2 binding partner" and can be a target peptide, e.g., angiotensin I, a kinin, or kinetensin or an analog thereof or a portion thereof, so long as the analog or portion of the target peptide is capable of binding to an ACE-2 polypeptide and optionally of being cleaved by an ACE-2 polypeptide. An ACE-2 binding partner can also be a polypeptide which is not a target peptide and which may, e.g., interact with an ACE-2 protein at sites other than the catalytic site.

The compounds of the invention can be identified using various assays depending on the type of compound and activity of the compound that is desired. Set forth below are at least some assays that can be used for identifying ACE-2 therapeutics. It is within the skill of the art to design additional assays for identifying ACE-2 therapeutics.

4.7.1 Cell-free assays

Cell-free assays can be used to identify compounds which are capable of interacting with an ACE-2 protein or binding partner, to thereby modify the activity of the ACE-2 protein or binding partner. Such a compound can, e.g., modify the structure of an ACE-2 protein or binding partner and thereby effect its activity. Cell-free assays can also be used to identify compounds which modulate the interaction between an ACE-2 protein and an ACE-2 binding partner, such as a target peptide. In a preferred embodiment, cell-free assays for identifying such compounds consist essentially in a reaction mixture containing an ACE-2 protein and a test compound or a library of test compounds in the presence or absence of a binding partner. A preferred binding partner is angiotensin I or kinetensin or portions thereof sufficient for interacting with ACE-2. A test compound can be, e.g., a derivative of an ACE-2 binding partner, e.g., an biologically inactive target peptide, or a small molecule.

Accordingly, one exemplary screening assay of the present invention includes the steps of contacting an ACE-2 protein or functional fragment thereof or an ACE-2 binding partner with a test compound or library of test compounds and detecting the formation of complexes. For detection purposes, the molecule can be labeled with a specific marker and the test compound or library of test compounds labeled with a different marker. Interaction of a test compound with an ACE-2 protein or fragment thereof or ACE-2 binding partner can then be detected by determining the level of the two labels after an incubation step and a washing step. The presence of two labels after the washing step is indicative of an interaction.

An interaction between molecules can also be identified by using real-time BIA (Biomolecular Interaction Analysis, Pharmacia Biosensor AB) which detects surface plasmon resonance (SPR), an optical phenomenon. Detection depends on changes in the mass concentration of macromolecules at the biospecific interface, and does not require any labeling of interactants. In one embodiment, a library of test compounds can be immobilized on a sensor surface, e.g., which forms one wall of a micro-flow cell. A solution containing the ACE-2 protein, functional fragment thereof, ACE-2 analog or ACE-2 binding partner is then flown continuously over the sensor surface. A change in the resonance angle as shown on a signal recording, indicates that an interaction has occurred. This technique is further described, e.g., in BIA technology Handbook by Pharmacia.

Another exemplary screening assay of the present invention includes the steps of (a) forming a reaction mixture including: (i) an ACE-2 polypeptide, (ii) an ACE-2 binding partner (e.g., angiotensin I, a kinin, or kinetensin), and (iii) a test compound; and (b)

detecting interaction of the ACE-2 and the ACE-2 binding protein. The ACE-2 polypeptide and ACE-2 binding partner can be produced recombinantly, purified from a source, e.g., plasma, or chemically synthesized, as described herein. A statistically significant change (potentiation or inhibition) in the interaction of the ACE-2 and ACE-2 binding protein in the presence of the test compound, relative to the interaction in the absence of the test compound, indicates a potential agonist (mimetic or potentiator) or antagonist (inhibitor) of ACE-2 bioactivity for the test compound. The compounds of this assay can be contacted simultaneously. Alternatively, an ACE-2 protein can first be contacted with a test compound for an appropriate amount of time, following which the ACE-2 binding partner is added to the reaction mixture. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified ACE-2 polypeptide or binding partner is added to a composition containing the ACE-2 binding partner or ACE-2 polypeptide, and the formation of a complex is quantitated in the absence of the test compound.

Complex formation between an ACE-2 protein and an ACE-2 binding partner may be detected by a variety of techniques. Modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled ACE-2 proteins or ACE-2 binding partners, by immunoassay, or by chromatographic detection.

Typically, it will be desirable to immobilize either ACE-2 or its binding partner to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of ACE-2 to an ACE-2 binding partner, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/ACE-2 (GST/ACE-2) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the ACE-2 binding partner, e.g. an <sup>35</sup>S-labeled ACE-2 binding partner, and the test compound, and the mixture incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes can be dissociated from the

matrix, separated by SDS-PAGE, and the level of ACE-2 protein or ACE-2 binding partner found in the bead fraction quantitated from the gel using standard electrophoretic techniques such as described in the appended examples.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, either ACE-2 or its cognate binding partner can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated ACE-2 molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with ACE-2 can be derivatized to the wells of the plate, and ACE-2 trapped in the wells by antibody conjugation. As above, preparations of an ACE-2 binding protein and a test compound are incubated in the ACE-2 presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the ACE-2 binding partner, or which are reactive with ACE-2 protein and compete with the binding partner; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the binding partner, either intrinsic or extrinsic activity. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the ACE-2 binding partner. To illustrate, the ACE-2 binding partner can be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. 3,3'-diamino-benzadine tetrahydrochloride or 4-chloro-1-naphthol. Likewise, a fusion protein comprising the polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) J Biol Chem 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as anti-ACE-2 antibodies, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the ACE-2 sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) J Biol Chem 266:21150-21157)

which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

Cell-free assays can also be used to identify compounds which interact with an ACE-2 protein and modulate an activity of an ACE-2 protein. Accordingly, in one embodiment, an ACE-2 protein is contacted with a test compound and the catalytic activity of ACE-2 is monitored. In one embodiment, the ability of ACE-2 to bind to and/or to hydrolyze a target peptide, e.g. angiotensin I, a kinin, such as bradykinin, or kinetensin, is determined. The binding affinity of ACE-2 to a target peptide can be determined according to methods known in the art. Determination of the enzymatic activity of ACE-2 can be performed as further described herein. In particular, enzymatic activity can be demonstrated by subjecting a reaction mixture containing an ACE-2 enzyme and a target peptide after incubation, to mass spectrometry, as described in the Examples.

In a preferred embodiment, the invention provides a screening method which comprises combining an ACE-2 polypeptide and a target peptide together with a test compound in a reaction mixture in conditions sufficient for the ACE-2 polypeptide to cleave the target peptide in the absence of the test compound. The method further comprises monitoring the presence of the target peptide, the target peptide conversion product, and/or of the one or more amino acids cleaved from the target peptide, such that a difference in the amount of at least one of the target peptide, the target peptide conversion product or one or more amino acids in the reaction mixture incubated with the test compound relative to a reaction mixture that does not contain the test compound indicates that the test compound is an ACE-2 therapeutic. In an even more preferred embodiment of the invention, the presence and/or the amount of the target peptide, the target peptide conversion product, and/or of the one or more amino acids in the reaction mixture is determined by spectrometric analysis of the reaction mixture or of a part thereof, e.g., as described in the Examples.

Assays can also be developed for identifying compounds which interact and optionally inhibit (i) ACE-2, but not ACE; (ii) ACE, but not ACE-2; and (iii) ACE-2 and ACE. This can be done by a two step screening assay, wherein each step can be conducted as described herein.

#### 4.7.2. Cell based assays

In addition to cell-free assays, such as described above, ACE-2 proteins as provided by the present invention, facilitate the generation of cell-based assays, e.g., for identifying small molecule agonists or antagonists. In one embodiment, a cell expressing an



ACE-2 protein on the outer surface of its cellular membrane is incubated in the presence of a test compound alone or a test compound and a molecule which is known to interact with ACE-2 and the interaction between ACE-2 and a test compound is detected, e.g., by using a microphysiometer (McConnell et al. (1992) Science 257:1906). An interaction between the ACE-2 protein the test compound is detected by the microphysiometer as a change in the acidification of the medium.

Cell based assays can also be used to identify compounds which modulate expression of an ACE-2 gene, modulate translation of an ACE-2 mRNA, or which modulate the stability of an ACE-2 mRNA or protein. Accordingly, in one embodiment, a cell which is capable of producing ACE-2, e.g., a kidney cell, is incubated with a test compound and the amount of ACE-2 produced in the cell medium is measured and compared to that produced from a cell which has not been contacted with the test compound. The specificity of the compound vis a vis ACE-2 can be confirmed by various control analysis, e.g., measuring the expression of one or more control genes.

Compounds which can be tested include small molecules, proteins, and nucleic acids. In particular, this assay can be used to determine the efficacy of ACE-2 antisense molecules or ribozymes.

In another embodiment, the effect of a test compound on transcription of an ACE-2 gene is determined by transfection experiments using a reporter gene operatively linked to at least a portion of the promoter of an ACE-2 gene. A promoter region of a gene can be isolated, e.g., from a genomic library according to methods known in the art. The reporter gene can be any gene encoding a protein which is readily quantifiable, e.g, the luciferase or CAT gene, well known in the art.

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

#### 4.8. Predictive Medicine

The invention further features predictive medicines, which are based, at least in part, on the identity of the novel ACE-2 genes and alterations in the genes and related pathway genes, which affect the expression level and/or function of the encoded ACE-2 protein in a subject.

For example, information obtained using the diagnostic assays (Diagnomics™ molecular diagnostics) described herein (alone or in conjunction with information on another genetic defect, which contributes to the same disease) is useful for diagnosing or confirming that a symptomatic subject (e.g. a subject symptomatic for hypertension, hypotension, CHF,

or a kinetensin associated condition), has a genetic defect (e.g. in an ACE-2 gene or in a gene that regulates the expression of an ACE-2 gene), which causes or contributes to the particular disease or disorder. Alternatively, the information (alone or in conjunction with information on another genetic defect, which contributes to the same disease) can be used prognostically for predicting whether a non-symptomatic subject is likely to develop a disease or condition, which is caused by or contributed to by an abnormal ACE-2 activity or protein level (e.g. hypertension, hypotension, CHF, or a kinetensin associated condition) in a subject. Based on the prognostic information, a doctor can recommend a regimen (e.g. diet or exercise) or therapeutic protocol, useful for preventing or prolonging onset of the particular disease or condition in the individual.

In addition, knowledge of the particular alteration or alterations, resulting in defective or deficient ACE-2 genes or proteins in an individual (the ACE-2 genetic profile), alone or in conjunction with information on other genetic defects contributing to the same disease (the genetic profile of the particular disease) allows customization of therapy for a particular disease to the individual's genetic profile, the goal of "pharmacogenomics". For example, an individual's ACE-2 genetic profile or the genetic profile of a disease or condition, to which ACE-2 genetic alterations cause or contribute, can enable a doctor: 1) to more effectively prescribe a drug that will address the molecular basis of the disease or condition; and 2) to better determine the appropriate dosage of a particular drug. For example, the expression level of ACE-2 proteins, alone or in conjunction with the expression level of other genes, known to contribute to the same disease, can be measured in many patients at various stages of the disease to generate a transcriptional or expression profile of the disease. Expression patterns of individual patients can then be compared to the expression profile of the disease to determine the appropriate drug and dose to administer to the patient.

The ability to target populations expected to show the highest clinical benefit, based on the ACE-2 or disease genetic profile, can enable: 1) the repositioning of marketed drugs with disappointing market results; 2) the rescue of drug candidates whose clinical development has been discontinued as a result of safety or efficacy limitations, which are patient subgroup-specific; and 3) an accelerated and less costly development for drug candidates and more optimal drug labeling (e.g. since the use of ACE-2 as a marker is useful for optimizing effective dose).

These and other methods are described in further detail in the following sections.

4.8.1. Prognostic and Diagnostic Assays

The present methods provide means for determining if a subject has (diagnostic) or is at risk of developing (prognostic) a disease, condition or disorder that is associated with an aberrant ACE-2 activity, e.g., an aberrant level of ACE-2 protein or an aberrant bioactivity. As set forth below, diseases or conditions that can be caused by or contributed to an abnormal ACE-2 level or bioactivity include diseases or conditions, which are caused by or contributed to by an abnormal amount of a target peptide of ACE-2 or level of angiotensin or kinetensin conversion product, resulting, e.g., from inappropriate hydrolysis. For example, the target peptide can be angiotensin I and the disease or condition associated with an aberrant level of an angiotensin conversion product, e.g., angiotensin II level (in this case abnormally high level) can be hypertension or congestive heart failure. In fact, in at least some tissues, an abnormally high angiotensin II level could result from an abnormally low activity of an ACE-2 enzyme, thereby allowing most or all of the angiotensin I to be converted into angiotensin II, instead of Ang.(1-9). Diseases associated with an abnormal kinetensin or kinetensin conversion product include histamine associated conditions such as allergies. The target peptide can also be a kinin, and the diseases or conditions associated with an aberrant kinin level (in this case an abnormally high kinin level) include inflammatory diseases, and pain. Furthermore, an abnormally high ACE-2 mRNA level has been found in cardiac tissue from individuals which had CHF (see Examples). Similarly, ACE has been found to be present at increased levels in the free wall, septum and apex of the hypertrophied left ventricle (Schunkert et al. (1990) J. Clin. Invest. 86:1913). Thus, it is believed that an abnormally high level of ACE-2 correlates with heart failure. Accordingly, the invention provides methods for determining whether a subject has or is likely to develop hypertension, hypotension, congestive heart failure, or a kinetensin associated condition, for example, comprising determining the level of an ACE-2 gene or protein, an ACE-2 bioactivity and/or the presence of a mutation or particular polymorphic variant in the ACE-2 gene. The invention also provides methods for determining whether the pain of a subject is caused by an abnormally low level of ACE-2.

Since ACE-2 is likely to catalyze hydrolysis of yet other peptides, e.g., neuropeptides such as substance P, the invention also provides methods for diagnosing, for example, neurological diseases or other diseases caused by inappropriate levels of ACE-2 target peptides.

In one embodiment, the method comprises determining whether a subject has an abnormal mRNA and/or protein level of ACE-2, such as by Northern blot analysis, reverse transcription-polymerase chain reaction (RT-PCR), *in situ* hybridization,

immunoprecipitation, Western blot hybridization, or immunohistochemistry. According to the method, cells are obtained from a subject and the ACE-2 protein or mRNA level is determined and compared to the level of ACE-2 protein or mRNA level in a healthy subject. An abnormal level of ACE-2 polypeptide or mRNA level is likely to be indicative of an aberrant ACE-2 activity.

In another embodiment, the method comprises measuring at least one activity of ACE-2. For example, the catalytic activity of ACE-2, e.g., capability to catalyze hydrolysis of certain peptides, e.g., angiotensin and/or kinins, can be determined, e.g., as described herein. Similarly, the constant of affinity of an ACE-2 protein of a subject with a target peptide, e.g. angiotensin or a kinin, can be determined. Comparison of the results obtained with results from similar analysis performed on ACE-2 proteins from healthy subjects will be indicative of whether a subject has an abnormal ACE-2 activity. Measurement of Ang.(1-9), Ang.(1-5), and/or kinetensin (1-8), level can also be used as an indicator of the activity of an ACE-2 enzyme in a subject. Such measurements can be done by spectrometry, as described herein.

In preferred embodiments, the methods for determining whether a subject has or is at risk for developing a disease associated with an aberrant ACE-2 activity is characterized as comprising detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one of (i) an alteration affecting the integrity of a gene encoding an ACE-2 polypeptide, or (ii) the mis-expression of the ACE-2 gene. To illustrate, such genetic alterations can be detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from an ACE-2 gene, (ii) an addition of one or more nucleotides to an ACE-2 gene, (iii) a substitution of one or more nucleotides of an ACE-2 gene, (iv) a gross chromosomal rearrangement of an ACE-2 gene, (v) a gross alteration in the level of a messenger RNA transcript of an ACE-2 gene, (vi) aberrant modification of an ACE-2 gene, such as of the methylation pattern of the genomic DNA, (vii) the presence of a non-wild type splicing pattern of a messenger RNA transcript of an ACE-2 gene, (viii) a non-wild type level of an ACE-2 polypeptide, (ix) allelic loss of an ACE-2 gene, and/or (x) inappropriate post-translational modification of an ACE-2 polypeptide. As set out below, the present invention provides a large number of assay techniques for detecting alterations in an ACE-2 gene. These methods include, but are not limited to, methods involving sequence analysis, Southern blot hybridization, restriction enzyme site mapping, and methods involving detection of absence of nucleotide pairing between the nucleic acid to be analyzed and a probe. These and other methods are further described *infra*.

Specific diseases or disorders, e.g., genetic diseases or disorders, are associated with specific allelic variants of polymorphic regions of certain genes, which do not necessarily encode a mutated protein. Thus, the presence of a specific allelic variant of a polymorphic region of a gene, such as a single nucleotide polymorphism ("SNP"), in a subject can render the subject susceptible to developing a specific disease or disorder. Polymorphic regions in genes, e.g. ACE-2 genes, can be identified, by determining the nucleotide sequence of genes in populations of individuals. If a polymorphic region, e.g., SNP is identified, then the link with a specific disease can be determined by studying specific populations of individuals, e.g. individuals which developed a specific disease, such as hypertension or CHF. A polymorphic region can be located in any region of a gene, e.g., exons, in coding or non coding regions of exons, introns, and promoter region.

It is likely that ACE-2 genes comprise polymorphic regions, specific alleles of which may be associated with specific diseases or conditions or with an increased likelihood of developing such diseases or conditions. In fact, allelic variants of the human ACE gene have been described and in some cases allelic variants have been found to be strongly associated with a higher risk for acute coronary events, sudden cardiac death, vascular restenosis after angioplasty, and idiopathic and hypertrophic cardiomyopathy (Malik et al. (1997) Am Heart J. 134:514). Thus, the invention provides methods for determining the identity of the allele or allelic variant of a polymorphic region of an ACE-2 gene in a subject, to thereby determine whether the subject has or is at risk of developing a disease or disorder associated with a specific allelic variant of a polymorphic region.

In an exemplary embodiment, there is provided a nucleic acid composition comprising a nucleic acid probe including a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence of an ACE-2 gene or naturally occurring mutants thereof, or 5' or 3' flanking sequences or intronic sequences naturally associated with the subject ACE-2 genes or naturally occurring mutants thereof. The nucleic acid of a cell is rendered accessible for hybridization, the probe is contacted with the nucleic acid of the sample, and the hybridization of the probe to the sample nucleic acid is detected. Such techniques can be used to detect alterations or allelic variants at either the genomic or mRNA level, including deletions, substitutions, etc., as well as to determine mRNA transcript levels.

A preferred detection method is allele specific hybridization using probes overlapping the mutation or polymorphic site and having about 5, 10, 20, 25, or 30 nucleotides around the mutation or polymorphic region. In a preferred embodiment of the invention, several probes capable of hybridizing specifically to allelic variants, such as single nucleotide polymorphisms, are attached to a solid phase support, e.g., a "chip".

Oligonucleotides can be bound to a solid support by a variety of processes, including lithography. For example a chip can hold up to 250,000 oligonucleotides. Mutation detection analysis using these chips comprising oligonucleotides, also termed "DNA probe arrays" is described e.g., in Cronin et al. (1996) *Human Mutation* 7:244. In one embodiment, a chip comprises all the allelic variants of at least one polymorphic region of a gene. The solid phase support is then contacted with a test nucleic acid and hybridization to the specific probes is detected. Accordingly, the identity of numerous allelic variants of one or more genes can be identified in a simple hybridization experiment.

In certain embodiments, detection of the alteration comprises utilizing the probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligase chain reaction (LCR) (see, e.g., Landegran et al. (1988) *Science* 241:1077-1080; and Nakazawa et al. (1994) *PNAS* 91:360-364), the latter of which can be particularly useful for detecting point mutations in the ACE-2 gene (see Abravaya et al. (1995) *Nuc Acid Res* 23:675-682). In a merely illustrative embodiment, the method includes the steps of (i) collecting a sample of cells from a patient, (ii) isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, (iii) contacting the nucleic acid sample with one or more primers which specifically hybridize to an ACE-2 gene under conditions such that hybridization and amplification of the ACE-2 gene (if present) occurs, and (iv) detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J.C. et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, D.Y. et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. et al., 1988, *Bio/Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In a preferred embodiment of the subject assay, mutations in, or allelic variants, of an ACE-2 gene from a sample cell are identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes

are determined by gel electrophoresis. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

5 In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the ACE-2 gene and detect mutations by comparing the sequence of the sample ACE-2 with the corresponding wild-type (control) sequence. Exemplary sequencing reactions include those based on techniques developed by Maxim and Gilbert (*Proc. Natl Acad Sci USA* (1977) 74:560) or Sanger (Sanger et al (1977) *Proc. Nat. Acad. Sci* 74:5463). It is also contemplated that any of a variety of automated  
10 sequencing procedures may be utilized when performing the subject assays (*Biotechniques* (1995) 19:448), including sequencing by mass spectrometry (see, for example PCT publication WO 94/16101; Cohen et al. (1996) *Adv Chromatogr* 36:127-162; and Griffin et al. (1993) *Appl Biochem Biotechnol* 38:147-159). It will be evident to one skilled in the art that, for certain embodiments, the occurrence of only one, two or three of the nucleic acid  
15 bases need be determined in the sequencing reaction. For instance, A-track or the like, e.g., where only one nucleic acid is detected, can be carried out.

In a further embodiment, protection from cleavage agents (such as a nuclease, hydroxylamine or osmium tetroxide and with piperidine) can be used to detect mismatched bases in RNA/RNA or RNA/DNA or DNA/DNA heteroduplexes (Myers, et al. (1985) *Science* 230:1242). In general, the art technique of "mismatch cleavage" starts by providing  
20 heteroduplexes formed by hybridizing (labelled) RNA or DNA containing the wild-type ACE-2 sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to base pair mismatches between the control and sample  
25 strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on  
30 denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton et al (1988) *Proc. Natl Acad Sci USA* 85:4397; Saleeba et al (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or  
35 more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA

mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in ACE-2 cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu et al. (1994) *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on an ACE-2 sequence, e.g., a wild-type ACE-2 sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility will be used to identify mutations or the identity of the allelic variant of a polymorphic region in ACE-2 genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) *Proc Natl. Acad. Sci USA* 86:2766, see also Cotton (1993) *Mutat Res* 285:125-144; and Hayashi (1992) *Genet Anal Tech Appl* 9:73-79). Single-stranded DNA fragments of sample and control ACE-2 nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labelled or detected with labelled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) *Trends Genet* 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing agent gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys Chem* 265:12753).

Examples of other techniques for detecting point mutations or the identity of the allelic variant of a polymorphic region include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation or nucleotide



difference (e.g., in allelic variants) is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki et al. (1986) *Nature* 324:163); Saiki et al (1989) *Proc. Natl Acad. Sci USA* 86:6230). Such allele specific oligonucleotide hybridization techniques may be used to test one mutation or polymorphic region per reaction when oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations or polymorphic regions when the oligonucleotides are attached to the hybridizing membrane and hybridized with labelled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation or polymorphic region of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238. In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini et al (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

In another embodiment, identification of the allelic variant is carried out using an oligonucleotide ligation assay (OLA), as described, e.g., in U.S. Pat. No. 4,998,617 and in Landegren, U. et al., *Science* 241:1077-1080 (1988). The OLA protocol uses two oligonucleotides which are designed to be capable of hybridizing to abutting sequences of a single strand of a target. One of the oligonucleotides is linked to a separation marker, e.g., biotinylated, and the other is detectably labeled. If the precise complementary sequence is found in a target molecule, the oligonucleotides will hybridize such that their termini abut, and create a ligation substrate. Ligation then permits the labeled oligonucleotide to be recovered using avidin, or another biotin ligand. Nickerson, D. A. et al. have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson, D. A. et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 87:8923-8927 (1990). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA.

Several techniques based on this OLA method have been developed and can be used to detect specific allelic variants of a polymorphic region of an ACE-2 gene. For

example, U.S. Patent No. 5593826 discloses an OLA using an oligonucleotide having 3'-amino group and a 5'-phosphorylated oligonucleotide to form a conjugate having a phosphoramidate linkage. In another variation of OLA described in Tobe et al. ((1996)Nucleic Acids Res 24: 3728), OLA combined with PCR permits typing of two alleles in a single microtiter well. By marking each of the allele-specific primers with a unique hapten, i.e. digoxigenin and fluorescein, each OLA reaction can be detected by using hapten specific antibodies that are labeled with different enzyme reporters, alkaline phosphatase or horseradish peroxidase. This system permits the detection of the two alleles using a high throughput format that leads to the production of two different colors.

The invention further provides methods for detecting single nucleotide polymorphisms in an ACE-2 gene. Because single nucleotide polymorphisms constitute sites of variation flanked by regions of invariant sequence, their analysis requires no more than the determination of the identity of the single nucleotide present at the site of variation and it is unnecessary to determine a complete gene sequence for each patient. Several methods have been developed to facilitate the analysis of such single nucleotide polymorphisms.

In one embodiment, the single base polymorphism can be detected by using a specialized exonuclease-resistant nucleotide, as disclosed, e.g., in Mundy, C. R. (U.S. Pat. No.4,656,127). According to the method, a primer complementary to the allelic sequence immediately 3' to the polymorphic site is permitted to hybridize to a target molecule obtained from a particular animal or human. If the polymorphic site on the target molecule contains a nucleotide that is complementary to the particular exonuclease-resistant nucleotide derivative present, then that derivative will be incorporated onto the end of the hybridized primer. Such incorporation renders the primer resistant to exonuclease, and thereby permits its detection. Since the identity of the exonuclease-resistant derivative of the sample is known, a finding that the primer has become resistant to exonucleases reveals that the nucleotide present in the polymorphic site of the target molecule was complementary to that of the nucleotide derivative used in the reaction. This method has the advantage that it does not require the determination of large amounts of extraneous sequence data.

In another embodiment of the invention, a solution-based method is used for determining the identity of the nucleotide of a polymorphic site. Cohen, D. et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087). As in the Mundy method of U.S. Pat. No. 4,656,127, a primer is employed that is complementary to allelic sequences immediately 3' to a polymorphic site. The method determines the identity of the nucleotide of that site using labeled dideoxynucleotide derivatives, which, if complementary to the nucleotide of the polymorphic site will become incorporated onto the terminus of the primer.

An alternative method, known as Genetic Bit Analysis or GBA™ is described by Goelet, P. et al. (PCT Appln. No. 92/15712). The method of Goelet, P. et al. uses mixtures of labeled terminators and a primer that is complementary to the sequence 3' to a polymorphic site. The labeled terminator that is incorporated is thus determined by, and complementary to, the nucleotide present in the polymorphic site of the target molecule being evaluated. In contrast to the method of Cohen et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087) the method of Goelet, P. et al. is preferably a heterogeneous phase assay, in which the primer or the target molecule is immobilized to a solid phase.

Recently, several primer-guided nucleotide incorporation procedures for assaying polymorphic sites in DNA have been described (Komher, J. S. et al., Nucl. Acids. Res. 17:7779-7784 (1989); Sokolov, B. P., Nucl. Acids Res. 18:3671 (1990); Syvanen, A. -C., et al., Genomics 8:684-692 (1990); Kuppuswamy, M. N. et al., Proc. Natl. Acad. Sci. (U.S.A.) 88:1143-1147 (1991); Prezant, T. R. et al., Hum. Mutat. 1:159-164 (1992); Ugozzoli, L. et al., GATA 9:107-112 (1992); Nyren, P. et al., Anal. Biochem. 208:171-175 (1993)). These methods differ from GBA™ in that they all rely on the incorporation of labeled deoxynucleotides to discriminate between bases at a polymorphic site. In such a format, since the signal is proportional to the number of deoxynucleotides incorporated, polymorphisms that occur in runs of the same nucleotide can result in signals that are proportional to the length of the run (Syvanen, A. -C., et al., Amer.J. Hum. Genet. 52:46-59 (1993)).

For mutations that produce premature termination of protein translation, the protein truncation test (PTT) offers an efficient diagnostic approach (Roest, et. al., (1993) *Hum. Mol. Genet.* 2:1719-21; van der Lijst, et. al., (1994) *Genomics* 20:1-4). For PTT, RNA is initially isolated from available tissue and reverse-transcribed, and the segment of interest is amplified by PCR. The products of reverse transcription PCR are then used as a template for nested PCR amplification with a primer that contains an RNA polymerase promoter and a sequence for initiating eukaryotic translation. After amplification of the region of interest, the unique motifs incorporated into the primer permit sequential *in vitro* transcription and translation of the PCR products. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis of translation products, the appearance of truncated polypeptides signals the presence of a mutation that causes premature termination of translation. In a variation of this technique, DNA (as opposed to RNA) is used as a PCR template when the target region of interest is derived from a single exon.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid, primer set; and/or

antibody reagent described herein, which may be conveniently used, *e.g.*, in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving an ACE-2 polypeptide.

Any cell type or tissue may be utilized in the diagnostics described below. In a preferred embodiment a bodily fluid, *e.g.*, blood, is obtained from the subject to determine the presence of a mutation or the identity of the allelic variant of a polymorphic region of an ACE-2 gene. A bodily fluid, *e.g.*, blood, can be obtained by known techniques (*e.g.* venipuncture). Alternatively, nucleic acid tests can be performed on dry samples (*e.g.* hair or skin). For prenatal diagnosis, fetal nucleic acid samples can be obtained from maternal blood as described in International Patent Application No. WO91/07660 to Bianchi. Alternatively, amniocytes or chorionic villi may be obtained for performing prenatal testing.

When using RNA or protein to determine the presence of a mutation or of a specific allelic variant of a polymorphic region of an ACE-2 gene, the cells or tissues that may be utilized must express the ACE-2 gene. Preferred cells for use in these methods include kidney cells and cardiac cells (see Examples). Alternative cells or tissues that can be used, can be identified by determining the expression pattern of the specific ACE-2 gene in a subject, such as by Northern blot analysis.

Diagnostic procedures may also be performed *in situ* directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents may be used as probes and/or primers for such *in situ* procedures (see, for example, Nuovo, G.J., 1992, *PCR in situ* hybridization: protocols and applications, Raven Press, NY).

In addition to methods which focus primarily on the detection of one nucleic acid sequence, profiles may also be assessed in such detection schemes. Fingerprint profiles may be generated, for example, by utilizing a differential display procedure, Northern analysis and/or RT-PCR.

Antibodies directed against wild type or mutant ACE-2 polypeptides or allelic variant thereof, which are discussed above, may also be used in disease diagnostics and prognostics. Such diagnostic methods, may be used to detect abnormalities in the level of ACE-2 polypeptide expression, or abnormalities in the structure and/or tissue, cellular, or subcellular location of an ACE-2 polypeptide. Structural differences may include, for example, differences in the size, electronegativity, or antigenicity of the mutant ACE-2 polypeptide relative to the normal ACE-2 polypeptide. Protein from the tissue or cell type to be analyzed may easily be detected or isolated using techniques which are well known to one of skill in the art, including but not limited to western blot analysis. For a detailed

explanation of methods for carrying out Western blot analysis, see Sambrook et al, 1989, supra, at Chapter 18. The protein detection and isolation methods employed herein may also be such as those described in Harlow and Lane, for example, (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), which is incorporated herein by reference in its entirety.

This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorimetric detection. The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of ACE-2 polypeptides. *In situ* detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody of the present invention. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the ACE-2 polypeptide, but also its distribution in the examined tissue. Using the present invention, one of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Often a solid phase support or carrier is used as a support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

One means for labeling an anti-ACE-2 polypeptide specific antibody is via linkage to an enzyme and use in an enzyme immunoassay (EIA) (Voller, "The Enzyme Linked Immunosorbent Assay (ELISA)", *Diagnostic Horizons* 2:1-7, 1978, Microbiological Associates Quarterly Publication, Walkersville, MD; Voller, et al., *J. Clin. Pathol.* 31:507-520 (1978); Butler, *Meth. Enzymol.* 73:482-523 (1981); Maggio, (ed.) *Enzyme Immunoassay*, CRC Press, Boca Raton, FL, 1980; Ishikawa, et al., (eds.) *Enzyme Immunoassay*, Kigaku

Shoin, Tokyo, 1981). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect fingerprint gene wild type or mutant peptides through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., *Principles of Radioimmunoassays*, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as  $^{152}\text{Eu}$ , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

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Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Moreover, it will be understood that any of the above methods for detecting alterations in a gene or gene product or polymorphic variants can be used to monitor the course of treatment or therapy.

#### 4.8.2. Pharmacogenomics

Knowledge of the particular alteration or alterations, resulting in defective or deficient ACE-2 genes or proteins in an individual (the ACE-2 genetic profile), alone or in conjunction with information on other genetic defects contributing to the same disease (the genetic profile of the particular disease) allows a customization of the therapy for a particular disease to the individual's genetic profile, the goal of "pharmacogenomics". For example, subjects having a specific allele of an ACE-2 gene may or may not exhibit symptoms of a particular disease or be predisposed of developing symptoms of a particular disease. Further, if those subjects are symptomatic, they may or may not respond to a certain drug, e.g., a specific ACE-2 therapeutic, but may respond to another. Thus, generation of an ACE-2 genetic profile, (e.g., categorization of alterations in ACE-2 genes which are associated with the development of a particular disease), from a population of subjects, who are symptomatic for a disease or condition that is caused by or contributed to by a defective and/or deficient ACE-2 gene and/or protein (an ACE-2 genetic population profile) and comparison of an individual's ACE-2 profile to the population profile, permits the selection or design of drugs that are expected to be safe and efficacious for a particular patient or patient population (i.e., a group of patients having the same genetic alteration).

For example, an ACE-2 population profile can be performed, by determining the ACE-2 profile, e.g., the identity of ACE-2 genes, in a patient population having a disease, which is caused by or contributed to by a defective or deficient ACE-2 gene. Optionally, the ACE-2 population profile can further include information relating to the response of the population to an ACE-2 therapeutic, using any of a variety of methods, including, monitoring: 1) the severity of symptoms associated with the ACE-2 related disease, 2) ACE-2 gene expression level, 3) ACE-2 mRNA level, and/or 4) ACE-2 protein level. and (iii) dividing or categorizing the population based on the particular genetic alteration or alterations present

in its ACE-2 gene or an ACE-2 pathway gene. The ACE-2 genetic population profile can also, optionally, indicate those particular alterations in which the patient was either responsive or non-responsive to a particular therapeutic. This information or population profile, is then useful for predicting which individuals should respond to particular drugs, based on their individual ACE-2 profile.

In a preferred embodiment, the ACE-2 profile is a transcriptional or expression level profile and step (i) is comprised of determining the expression level of ACE-2 proteins, alone or in conjunction with the expression level of other genes, known to contribute to the same disease, can be measured in many patients at various stages of the disease.

Pharmacogenomic studies can also be performed using transgenic animals. For example, one can produce transgenic mice, e.g., as described herein, which contain a specific allelic variant of an ACE-2 gene. These mice can be created, e.g, by replacing their wild-type ACE-2 gene with an allele of the human ACE-2 gene. The response of these mice to specific ACE-2 therapeutics can then be determined.

#### 4.8.3. Monitoring of Effects of ACE-2 Therapeutics During Clinical Trials

The ability to target populations expected to show the highest clinical benefit, based on the ACE-2 or disease genetic profile, can enable: 1) the repositioning of marketed drugs with disappointing market results; 2) the rescue of drug candidates whose clinical development has been discontinued as a result of safety or efficacy limitations, which are patient subgroup-specific; and 3) an accelerated and less costly development for drug candidates and more optimal drug labeling (e.g. since the use of ACE-2 as a marker is useful for optimizing effective dose).

The treatment of an individual with an ACE-2 therapeutic can be monitored by determining ACE-2 characteristics, such as ACE-2 protein level or activity, ACE-2 mRNA level, ACE-2 transcriptional level, and/or level of one or more angiotensin or kinetensin conversion products, e.g., Ang.(1-9). This measurements will indicate whether the treatment is effective or whether it should be adjusted or optimized. Thus, ACE-2 can be used as a marker for the efficacy of a drug during clinical trials.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a preadministration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of an ACE-2 protein, mRNA, or genomic DNA and/or level



of one or more angiotensin or kinetensin conversion products, e.g., Ang.(1-9) in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the ACE-2 protein, mRNA, or genomic DNA and/or level of one or more angiotensin or kinetensin conversion products, e.g., Ang.(1-9) in the post-administration samples; (v) comparing the level of expression or activity of the ACE-2 protein, mRNA, or genomic DNA and/or level of one or more angiotensin or kinetensin conversion products, e.g., Ang.(1-9) in the preadministration sample with that in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of ACE-2 to higher levels than detected, i.e., to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of ACE-2 to lower levels than detected, i.e., to decrease the effectiveness of the agent.

Cells of a subject may also be obtained before and after administration of an ACE-2 therapeutic to detect the level of expression of genes other than ACE-2, to verify that the ACE-2 therapeutic does not increase or decrease the expression of genes which could be deleterious. This can be done, e.g., by using the method of transcriptional profiling. Thus, mRNA from cells exposed in vivo to an ACE-2 therapeutic and mRNA from the same type of cells that were not exposed to the ACE-2 therapeutic could be reverse transcribed and hybridized to a chip containing DNA from numerous genes, to thereby compare the expression of genes in cells treated and not treated with an ACE- therapeutic. If, for example an ACE-2 therapeutic turns on the expression of a proto-oncogene in an individual, use of this particular ACE-2 therapeutic may be undesirable.

#### 4.9. Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject having or likely to develop a disorder associated with aberrant ACE-2 expression or activity or aberrant level of angiotensin or kinetensin or one or more angiotensin or kinetensin conversion product, e.g., disorders or diseases associated with an abnormal blood pressure.

##### 4.9.1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant ACE-2 expression or activity or an abnormal amount of target peptide or abnormal blood pressure, by administering to the

subject an agent which modulates ACE-2 expression or at least one ACE-2 activity. Subjects at risk for such a disease can be identified by a diagnostic or prognostic assay, e.g., as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the ACE-2 aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of ACE-2 aberrancy, for example, a ACE-2 agonist or ACE-2 antagonist agent can be used for treating the subject prophylactically. In particular, an ACE-2 therapeutic can be administered prophylactically in a subject having elevated levels of angiotensin II, before any other symptoms are present. Such a prophylactic treatment could thus prevent the development of a abnormally high blood pressure. An agonist can also be administered prophylactically to prevent the development of a kinetensin associated condition, e.g., allergies. The prophylactic methods are similar to therapeutic methods of the present invention and are further discussed in the following subsections.

#### 4.9.2. Therapeutic Methods

In general, the invention provides methods for treating a disease caused by or contributed to by an aberrant ACE-2 activity or an abnormal blood pressure or abnormal amount of target peptide or angiotensin conversion product in a subject, comprising administering to the subject an effective amount of a pharmaceutical composition comprising a compound which is capable of modulating an ACE-2 activity, such that the disease is treated or prevented in the subject. Among the approaches which may be used to ameliorate disease symptoms involving an aberrant ACE-2 activity are, for example, antisense, ribozyme, and triple helix molecules described above. Examples of suitable compounds include the antagonists, agonists or homologues described in detail herein, as well as angiotensin conversion products or derivatives thereof.

#### 4.9.3. Diseases or Conditions that can be Treated or Prevented with ACE-2 therapeutics

As set forth herein, ACE-2 has significant sequence homologies with ACE enzymes and has also been shown to hydrolyze angiotensin I into Ang.(1-9) (see Example 5.4.). It has also been described that Ang.(1-9) can further be hydrolyzed by ACE into Ang.(1-5). Thus, ACE-2 competes with ACE for the substrate angiotensin I and hydrolyzes angiotensin I into Ang.(1-9), instead of angiotensin II (Ang.(1-8), a potent vasoconstrictor. Angiotensin I can also be converted into other peptides, having either vasoconstrictive or anti-hypertensive activity (e.g., Ang.(1-7), see, e.g., Chappel et al. (1998) Hypertension 31:

362). Various angiotensin conversion pathways are shown in Figure 7. Thus, based at least on the fact that ACE-2 has the same substrate as ACE and that both types of enzymes share significant sequence homologies in functional domains, ACE-2 therapeutics can be used for similar therapeutic and preventive purposes as ACE, as well as additional ones. ACE-2 therapeutics can thus be used not only for treating or preventing diseases or disorders associated with an aberrant ACE-2 activity, but also for treating or preventing diseases or disorders associated with an aberrant ACE activity or activity of any other enzyme that is involved in the conversion of a substrate of ACE-2, such as angiotensin I. For example, ACE-2 therapeutics can be useful for treating diseases associated with an abnormal Ang.(1-5), Ang.(1-7) level, or Ang.(1-8)(i.e., angiotensin II) level.

In certain embodiments, treating or preventing a disease in a subject includes administering to the subject an ACE-2 therapeutic which is also an ACE therapeutic or administering to the subject both an ACE-2 and an ACE therapeutic. For example, the ACE-2 therapeutic is an antagonist of both ACE-2 and ACE. Thus, administration of such a compound to a subject will result in inhibition of hydrolysis of angiotensin I into angiotensin II as well as into Ang.(1-9). Alternatively, the ACE-2 therapeutic can be an agonist of both ACE-2 or ACE.

The identity of the therapeutic compound for use in treating or preventing individual diseases or disorders can be determined by performing in vitro and/or in vivo assays. Preferred assays are those using animal models, such as animal models for hypertension. In an illustrative embodiment, a hypertensive rat of the transgenic rat strain TGR(mREN2)27 harboring the murine Ren-2 gene or a transgenic rat comprising a transgene encoding human angiotensinogen and/or human renin (U.S. Patent No. 5,731,489) are used. These transgenic rats develop fulminant hypertension at an early age (Lee et al. (1996) Am J Physiol 270: E919) and are thus useful for testing ACE-2 therapeutics. Such animal models can also be used for determining the effect of a specific angiotensin conversion product on a disorder, e.g., hypertension, and thereby allow to predict whether an agonist or an antagonist should be used for treating a specific disease or condition.

Accordingly, in a preferred embodiment, the compounds of the present invention are useful for regulating blood pressure and in particular arterial hypertension. The method comprises, e.g., administering to the subject an effective amount of a pharmaceutical composition comprising an ACE-2 therapeutic compound. An ACE-2 therapeutic compound can be, e.g., a compound which inhibits the formation of angiotensin II, a potent

vasoconstrictor, such as an ACE-2 agonist, which, e.g., hydrolyzes angiotensin I into Ang.(1-9).

Blood pressure refers to the pressure exerted by the blood upon the walls of the blood vessels, e.g., arteries, and is usually measured on the radial artery by means of a sphygmomanometer, and expressed in millimeters of mercury. The following ranges of blood pressure are usually used as a standard for normal versus abnormal blood pressure: a normal blood pressure corresponds to a diastolic blood pressure of less than 85 mm Hg; a high normal blood pressure corresponds to a diastolic blood pressure between 85 and 89 mm Hg; a mild hypertension corresponds to a diastolic blood pressure between 90-104 mm Hg; a moderate hypertension corresponds to a diastolic blood pressure between 105 and 114 mm Hg; and severe hypertension corresponds to a diastolic blood pressure higher than 115 mm Hg. Abnormal blood pressure can also be determined based on the systolic blood pressure (when the diastolic pressure is less than 90 mm Hg). Thus, a normal blood pressure corresponds to a systolic blood pressure of less than 140 mm Hg; a borderline systolic hypertension corresponds to a systolic blood pressure between 140 and 159 mm Hg; and isolated systolic hypertension corresponds to a systolic blood pressure higher than 160 mm Hg. This classification is borrowed from *Cecil: Essentials of Medicine*, Third Edition by Andreoli et al. W.B. Saunders Company (1993).

A diagnosis of hypertension, also referred to herein as "abnormally high blood pressure", is usually made in an adult over 18 years of age if the average of two or more blood pressure measurements on at least two subsequent visits is 90 mm Hg or higher diastolic or 140 mm Hg systolic. Since children and pregnant women have a lower blood pressure, a blood pressure over 120/80 (i.e., 120 mm Hg systolic blood pressure/80 mm Hg diastolic blood pressure), is considered abnormal. Isolated systolic hypertension (ISH) refers to a condition in which the systolic blood pressure is greater than 160 mm Hg and the diastolic blood pressure is less than 85 mm Hg. ISH is associated with enhanced morbidity.

ACE-2 therapeutics can also be used to treat other blood pressure related diseases or conditions, e.g., CHF, chronic heart failure, left ventricular hypertrophy, acute heart failure, myocardial infarction, and cardiomyopathy. In a preferred embodiment, ACE-2 therapeutics are used to treat CHF. CHF is characterized by the inability of the left ventricle to maintain a normal blood pressure. This results in a baroreflex-mediated reflex increase in sympathetic discharge, which stimulates the myocardium to beat faster and stronger, yet increases peripheral vasoconstriction so that the afterload rises and the load on the failing myocardium augments (Lionel H. Opie, *Drugs for the Heart*, Third Edition, W.B. Saunders Co., 1991). Excess adrenergic activity also results in enhanced activity of the renin-

angiotensin system, further increasing peripheral vascular resistance and contributing to fluid retention (edema) by stimulation of the secretion of aldosterone. In addition, angiotensin promotes the release of vasopressin to contribute to abnormal volume regulation and hyponatremia in severe CHF. Overloading of the left ventricle also results in hypertrophy of the ventricular muscle, resulting in a decrease in its contractility, further contributing to the condition. As described in the background section, vasodilators such as ACE-inhibitors are efficient in treating CHF and reducing mortality. ACE-inhibitors are particularly preferred therapeutics for treating CHF since they are able to inhibit the deleterious neurohumoral vicious circle involving angiotensin-renin-aldosterone. Thus, it is believed that ACE-2 therapeutics, which also modulate angiotensin hydrolysis, will also be useful for treating and preventing CHF.

In another embodiment, the invention provides methods for regulating cell proliferation, such as smooth cell proliferation. Smooth muscle cell proliferation in the intima of muscular arteries is a primary cause of vascular stenosis in atherosclerosis, after vascular surgery, and after coronary angioplasty. Several animal studies have indicated that the renin-angiotensin system plays an important role in this vascular response to injury. In particular, it has been shown that chronic treatment with ACE inhibitors reduced myointimal thickening following balloon injury in rat carotid artery or aorta (Powell et al. (1991) J. Am. Coll. Cardiol. 17:137B-42B). The stimulatory effect of angiotensin II on cell growth and replication in the cardiovascular system, which may result in myocardial hypertrophy and hypertrophy or hyperplasia of conduit and resistance vessels in certain subjects is mediated through angiotensin II receptors (subtype AT1) (Rosendorff C. (1996) J. Am. Coll. Cardiol. 28: 803). The importance of ACE in atherosclerosis is further described, e.g. in Malik et al. (1997) Am. Heart J. 134:514. It has also been shown, that angiotensin caused myocyte hypertrophy and fibroblast proliferation associated with the induction of mRNA for several early response genes (c-fos, c-jun, jun B, Egr-1 and c-myc), angiotensinogen and transforming growth factor beta (TGF $\beta$ ) (Rosendorff (1996) J. Am. Coll. Cardiol. 28: 803-12; Paquet et al. (1990) J. Hypertens.: 8: 565-72). Accordingly, in one embodiment, the invention provides a method for reducing or inhibiting smooth muscle cell proliferation, comprising administering to a subject an efficient amount of a composition comprising an ACE-2 therapeutic. In one embodiment, the therapeutic is administered systemically. However, the ACE-2 therapeutic can also be administered locally, e.g., at a site of vascular injury.

ACE-2 therapeutics can further be used in treating kidney diseases or disorders. Angiotensin and ACEs are important in the development and for the maintenance

of the functional and structural integrity of the adult kidney (see, e.g., Hilgers et al. (1997) Semin. Nephrol. 17:492). Chronic renal disease evolves to end-stage renal failure through events, including enhanced intraglomerular pressure and plasma protein ultrafiltration, mediated at least in part by angiotensin II. It has been reported that ACE inhibitors reduce intracapillary pressure and ameliorate glomerular size-selective function (see, e.g., Ruggenti and Remuzzi (1997) Curr. Opin. Nephrol. Hypertens. 6:489). Thus, based at least in part on the fact that ACE-2 is expressed in kidney and is homologous to ACE, ACE2-2 therapeutics can be used for treating and preventing renal diseases.

The role of angiotensin inhibitors in the release of norepinephrine from the terminal adrenergic neurons leads to the proposal that angiotensin inhibitors should be useful for treating various other hyperadrenergic states, such as acute myocardial infarction (AMI) and some ventricular arrhythmias.

The invention further provides methods for treating kinetensin associated conditions. As described herein, ACE-2 cleaves the C-terminal amino acid (leucine) from kinetensin. Kinetensin is a nine amino acid peptide having SEQ ID NO: 23 which has been reported to induce a dose-dependent release of histamine from mast cells, as well as induce a dose-dependent increase in vascular permeability when injected intradermally (Sydbom et al. (1989) *Agents Actions* 27: 68) into rats. Accordingly, modulating the plasma and/or tissue level of kinetensin, such as by modulating the hydrolysis of the C-terminal amino acid from kinetensin, should be useful for treating conditions that are caused by, or contributed to by, an abnormal kinetensin level. Such conditions include those caused by, or contributed to by, an abnormal histamine release from mast cells and/or by an abnormal vascular permeability. Since excessive histamine release is associated with local or systemic allergic reactions, including exzema, asthma, anaphylactic shock, ACE-2 therapeutics are believed to be useful for treating these conditions.

In another preferred embodiment, the invention provides a method for decreasing or inhibiting an inflammatory reaction. In fact, based at least in part on the homology between ACE and ACE-2 and the fact that ACE is capable of hydrolysing polypeptides other than angiotensin I, such as kinins, e.g., bradykinin, ACE-2 is likely to hydrolyze kinins. Kinins (e.g., bradykinin and kallidin) are generally involved in inflammation. In fact, part of the initiation process of an inflammatory reaction is mediated by peptide kinins, such as bradykinin, which are liberated by kallikrein proteases upon tissue destruction. The kinins or other peptide messengers, act on specific cell receptors at the

inflammation site to activate the phospholipase enzymes A2 and/or C, to initiate the arachidonate cascade.

Bradykinins are involved in inflammatory reactions on various tissues. For example, it has been found that bradykinin is produced in inflammatory reactions in the intestine, provoking contraction of smooth muscle and secretion of fluid and ions. The existence of specific bradykinin receptors in the mucosal lining of the intestine and in intestinal smooth muscle is demonstrated by Manning et al. (Nature 229: 256 (1982)), showing the influence of bradykinin in very low concentrations upon fluid and ion secretion. Thus, the invention can be used to treat inflammatory reactions in the intestine.

Similarly, the compounds of the present invention are also expected to be effective in treating other diseases or conditions such as SIRS (Systemic Inflammatory Response Syndromes)/sepsis, polytrauma, inflammatory bowel disease, acute and chronic pain, bone destruction in rheumatoid and osteo arthritis and periodontal disease, dysmenorrhea, premature labor, brain edema following focal injury, diffuse axonal injury, stroke, reperfusion injury and cerebral vasospasm after subarachnoid hemorrhage, allergic disorders including asthma, adult respiratory distress syndrome, wound healing and scar formation.

Furthermore, the invention also provides analgesic methods. In fact, bradykinin is known to be one of the most potent naturally occurring stimulators of C-fiber afferents mediating pain. The production of bradykinin results in pain at the site of the pathological condition, and the overproduction intensifies the pain directly or via stimulation by bradykinin of the activation of the arachidonic acid pathway which produces prostaglandins and leukotrienes, more distal mediators of inflammation (Handbook of Experimental Pharmacology, Vol.25, Springer-Verlag (1969), and Vol. 25 Supplement (1979); Stewart, in "Mediators of the Inflammatory Process," Henson and Murphy, eds., Elsevier,(1989)). For example, direct application of bradykinin to denuded skin or intra-arterial or visceral injection results in the sensation of pain in animals and in man. Kinin-like materials have been isolated from inflammatory sites produced by a variety of stimuli. In addition, bradykinin receptors have been localized to nociceptive peripheral nerve pathways and bradykinin has been demonstrated to stimulate central fibers mediating pain sensation. Bradykinin has also been shown to be capable of causing hyperalgesia in animal models of pain. (Burch et al., J. Med. Chem., 30:237-269 (1990) and Clark, W. G. Handbook of Experimental Pharmacology, Vol. XXV: Bradykinin, kallidin, and kallikrein. Erdo, E. G. (ed.), 311-322 (1979)) . Furthermore, a number of studies have demonstrated that bradykinin antagonists are capable of blocking or ameliorating both pain as well as

hyperalgesia in both animals and man (Ammons, W. S. et al. The American Physiological Society, 0363-6119 (1985); Clark, Handbook of Experimental Pharmacology, Vol. XXV: Bradykinin, kallidin, and kallikrein. Erdo, E. G. (ed.), 311-322 (1979); Costello al., European Journal of Pharmacology, 171:259-263 (1989); Laneuville et al. European Journal of Pharmacology, 137:281-285 (1987); Steranka et al, European Journal of Pharmacology, 16:261-262 (1987); and Steranka et al, Neurobiology, 85:3245-3249 (1987)). Similarly, Whalley et al, in Naunyn Schmiederberg's Arch. Pharmacol., 336:652-655 (1987) have demonstrated that bradykinin antagonists are capable of blocking bradykinin-induced pain in a human blister base model. Thus, the compositions of the invention comprising an ACE-2 agonist therapeutics can be applied topically to hydrolyze and thereby inactive bradykinin and/or related kinins to thereby inhibit or reduce pain in burned skin, e.g. in severely burned patients in whom large doses of narcotics are required over long periods of time and for the local treatment of relatively minor burns or other forms of local skin injury.

Similarly, the production of bradykinin seems to be associated with the pain in angina and myocardial ischemia (Kimura et al., Amer. Heart J. 85: 635 (1973); Staszewska-Barczak et al., Cardiovasc. Res. 10: 314 (1976)). Thus, ACE-2 therapeutics could be used to relieve pain in subjects suffering from angina or myocardial ischemia by degradation of bradykinin.

The use of the compounds of the invention for reducing or inhibiting pain have significant advantages over currently accepted therapeutic approaches to analgesia. In fact, while mild to moderate pain can be alleviated with the use of nonsteroidal anti-inflammatory drugs and other mild analgesics, severe pain such as that accompanying surgical procedures, burns and severe trauma requires the use of narcotic analgesics. These drugs carry the limitations of abuse, potential, physical and psychological dependence, altered mental status and respiratory depression which significantly limit their usefulness. On the contrary, the compounds of the invention, i.e., ACE-2 agonist therapeutics, are likely to be devoid of such undesirable secondary effects.

Other disease states in which ACE-2 agonist therapeutics can be useful include in the treatment of burns, perioperative pain, migraine and other forms of pain, shock, central nervous system injury, rhinitis, premature labor, etc. Yet other diseases or conditions in which bradykinin is overproduced and in which ACE-2 agonist therapeutics capable of inactivating bradykinin can be useful include pathological conditions such as septic (Robinson et al., Am. J. Med. 59: 61 (1975)) and hemorrhagic (Hirsch et al., J. Surg. Res. 17: 147 (1974)) shock, anaphylaxis (Collier and James, J. Physiol. 160: 15P (1966)), arthritis (Jasani et al., Ann. Rheum. Dis. 28: 497 (1969); Hamberg et al., Agents Actions 8:



50( 1978); Sharma et al., Arch. Int. Pharmacodyn. 262: 279 (1983)), rhinitis (Proud et al., J. Clin. Invest. 72: 1678 (1983); Naclerio et al., Clin. Res. 33: 613A (1985)), asthma (Christiansen et al., J. Clin. Invest. 79: 188 (1987)), inflammatory bowel disease (Zeitlin and Smith, Gut 14: 133 (1973)), sarcoidosis (*Cecil: Essentials of Medicine*, Third Edition by Andreoli et al. W.B. Saunders Company (1993)), and certain other conditions including acute pancreatitis, post-gastrectomy dumping syndrome, carcinoid syndrome, migraine, and hereditary angioedema.

Furthermore, bradykinin and bradykinin-related kinins are not only produced endogenously, but may also be injected into an animal, e.g., a human, via stings or bites. It is known that insects such as hornets and wasps inject bradykinin related peptides that cause pain, swelling and inflammation. Accordingly, the method provides methods and compounds for treating insect stings or bites, comprising administering either locally or systemically to a subject having an insect bite or sting an ACE-2 agonist therapeutic, to thereby relieve the pain and reduce the inflammation.

Based at least on the presence of ACE-2 in testis, ACE- therapeutics could also have a utility in treating infertility or other disorders relating to gamete maturation.

In addition, it has been discovered that ACE inhibitors are useful in treating cognitive disorders [German Application No. 3901-291-A, published Aug. 3, 1989]. Accordingly, ACE-2 antagonists of the invention are also likely to be useful in treating cognitive disorders.

#### 4.9.4. Effective Dose

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining The Ld<sub>50</sub> (The Dose Lethal To 50% Of The Population) And The Ed<sub>50</sub> (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>. Compounds which exhibit large therapeutic induces are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form

employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC<sub>50</sub> (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

#### 4.9.5. Formulation and Use

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by, for example, injection, inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For such therapy, the compounds of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the compounds of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents

(e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., ationd oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner. For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and

include, for example, for transmucosal administration bile salts and fusidic acid derivatives. in addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally known in the art. A wash solution can be used locally to treat an injury or inflammation to accelerate healing.

In clinical settings, a gene delivery system for the therapeutic ACE-2 gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g., by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g., Chen et al. (1994) PNAS 91: 3054-3057). An ACE-2 gene, such as any one of the sequences represented in the group consisting of SEQ ID NOS 1 and 3 or a sequence homologous thereto can be delivered in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) Cancer Treat Rev 20:105-115).

The pharmaceutical preparation of the gene therapy construct or compound of the invention can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle or compound is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

#### 4.10. Kits

The invention further provides kits for use in diagnostics or prognostic methods or for treating a disease or condition associated with an aberrant ACE-2 activity, ACE-2 target peptide level, or angiotensin or kinetensin conversion product level. The

invention also provides kits for determining which ACE-2 therapeutic should be administered to a subject. The invention encompasses kits for detecting the presence of ACE-2 mRNA or protein in a biological sample or for determining the presence of mutations or the identity of polymorphic regions in an ACE-2 gene. For example, the kit can comprise a labeled compound or agent capable of detecting ACE-2 protein or mRNA in a biological sample; means for determining the amount of ACE-2 in the sample; and means for comparing the amount of ACE-2 in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect ACE-2 mRNA or protein.

In one embodiment, the kit comprises a pharmaceutical composition containing an effective amount of an ACE-2 therapeutic and instruction for use in treating or preventing hypertension. In another embodiment, the kit comprises a pharmaceutical composition comprising an effective amount of an ACE-2 agonist therapeutic and instructions for use in treating insect bites. Generally, the kit comprises a pharmaceutical composition comprising an effective amount of an ACE-2 agonist or antagonist therapeutic and instructions for use as an analgesic.

Yet other kits can be used to determine whether a subject has or is likely to develop a disease or condition associated with an aberrant ACE-2 activity. Such a kit can comprise, e.g., one or more nucleic acid probes capable of hybridizing specifically to at least a portion of an ACE-2 gene or allelic variant thereof, or mutated form thereof.

#### 4.11. Additional Uses for ACE-2 Proteins and Nucleic Acids

The ACE-2 nucleic acids of the invention can further be used in the following assays. In one embodiment, the human ACE-2 nucleic acid having SEQ ID NO:1 or a portion thereof, or a nucleic acid which hybridizes thereto can be used to determine the chromosomal localization of an ACE-2 gene. Comparison of the chromosomal location of the ACE-2 gene with the location of chromosomal regions which have been shown to be associated with specific diseases or conditions, e.g., by linkage analysis (coinheritance of physically adjacent genes), can be indicative of diseases or conditions in which ACE-2 may play a role. A list of chromosomal regions which have been linked to specific diseases can be found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library) and at <http://www3.ncbi.nlm.nih.gov/Omim/> (Online Mendelian Inheritance in Man). Furthermore, the ACE-2 gene can also be used as a chromosomal marker in genetic linkage studies involving genes other than ACE-2.

Chromosomal localization of a gene can be performed by several methods well known in the art. For example, Southern blot hybridization or PCR mapping of somatic cell hybrids can be used for determining on which chromosome or chromosome fragment a specific gene is located. Other mapping strategies that can similarly be used to localize a gene to a chromosome or chromosomal region include in situ hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Furthermore, fluorescence in situ hybridization (FISH) of a nucleic acid, e.g., an ACE-2 nucleic acid, to a metaphase chromosomal spread is a one step method that provides a precise chromosomal location of the nucleic acid. This technique can be used with nucleic acids as short as 500 or 600 bases; however, clones larger than 2,000 bp have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Such techniques are described, e.g. in Verma et al., *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988). Using such techniques, a gene can be localized to a chromosomal region containing from about 50 to about 500 genes.

If the ACE-2 gene is shown to be localized in a chromosomal region which cosegregates, i.e., which is associated, with a specific disease, the differences in the cDNA or genomic sequence between affected and unaffected individuals are determined. The presence of a mutation in some or all of the affected individuals but not in any normal individuals, will be indicative that the mutation is likely to be causing or contributing to the disease.

The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent applications as cited throughout this application are hereby expressly incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2<sup>nd</sup> Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells*

And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical  
5 Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

10      5.              Examples

5.1.              Cloning and Analysis of Human ACE-2

A full length cDNA encoding human ACE-2 was isolated as follows. A cDNA library was prepared from a human heart of a subject who had congestive heart failure. Random sequencing of clones from the library identified a 1.6 kb clone that has homology  
15 to human angiotensin converting enzyme, and which is thus referred to as ACE-2.

5' RACE was used to clone the 5' end of the ACE-2 gene. RACE was performed using Clontech's Marathon cDNA Amplification Kit. First strand cDNA synthesis was performed using the cDNA synthesis primer supplied with the kit and 1 µg polyA<sup>+</sup> RNA prepared from the heart of a 43 year old woman with an idiopathic  
20 cardiomyopathy using 100 u MMLV reverse transcriptase. Second strand cDNA synthesis was then performed using the second strand enzyme cocktail of the Clontech kit. The Marathon cDNA adaptor was ligated to the double stranded cDNA with T4 DNA ligase. A gene specific primer was designed starting about 400 bp downstream of the 5' end of the 1.6 kb ACE-2 clone. The primer had the nucleotide sequence 5' CAC AGG TTC CAC CAC  
25 CCC AAC TAT CTC 3' (SEQ ID NO: 13, which corresponds to nucleotides 1528-1554 of SEQ ID NO: 1) and the hybridization temperature (T<sub>m</sub>) used was 62.7 °C (GC content of 55%). The gene specific primer and an adaptor primer were used for 5' RACE using the Advantage KlenTaq polymerase mix under the following conditions: 1 cycle at 94 °C for 2 minutes; 35 cycles of 94 °C for 30 sec., 60 °C for 45 sec., 72 °C for 30 sec.; and 1 cycle at  
30 72 °C for 5 minutes. A comparison of the partial ACE-2 clone with the previously cloned ACE genes indicated that about 1000 bp were necessary to have the full length clone if both genes have a similar length. RACE products of the expected size were obtained. These products were Southern blotted and shown to hybridize with a probe, corresponding to nucleotides 1152 to 1318 of SEQ ID NO: 1, from the partial ACE-2 clone. The RACE  
35 products were run on a 1.2% agarose gel, the expected size fragments were visualized,

excised and purified using the Jetsorb Gel Extraction kit (Genomed). The fragments were then ligated into the TA cloning vector pCR2.1 (InVitrogen). A clone containing a 1.6 kb insert was sequenced and found to have the 5' end of the gene.

5 A full length ACE encoding DNA was then prepared as follows. An EcoRI-BamHI fragment of the 5' RACE clone and a BamHI-NotI fragment of the partial clone containing the 3' end of the ACE-2 cDNA described above were ligated into the EcoRI-NotI sites of pCDNA3.1+ vector (InVitrogen). The clones were analyzed by restriction mapping and sequencing. This confirmed that full length cDNA clones encoding human ACE were obtained.

10 The cDNA described herein encoding ACE-2 is 3396 nucleotides long and has the nucleotide sequence shown in Figure 1 and set forth in SEQ ID NO: 1. A nucleic acid comprising this cDNA has been deposited at the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD) on December 3, 1997 has been assigned ATCC Designation No. 209510. This cDNA has an open reading frame from nucleotide 82 to  
15 nucleotide 2496 of SEQ ID NO: 1 which is set forth in SEQ ID NO: 3 and encodes a protein of 805 amino acids having the amino acid sequence shown in Figure 1 and set forth in SEQ ID NO: 2. The ACE-2 protein having SEQ ID NO: 2 contains a hydrophobic signal sequence from amino acid 1 to amino acid 18. Thus, the mature ACE-2 protein has the amino acid sequence from about amino acid 19 to amino acid 805 of SEQ ID NO: 2. The  
20 presence of the signal peptide indicates that the ACE-2 protein is secreted and/or membrane bound.

The ACE-2 protein further comprises a Zinc binding domain, from about amino acid 374 to amino acid 378 of SEQ ID NO: 2 which is encoded by the nucleotide sequence from nucleotide 1201 to nucleotide 1215 of SEQ ID NO: 1. The ACE-2 protein  
25 also comprises a hydrophobic transmembrane domain corresponding to amino acids 741 to amino acid 765 of SEQ ID NO: 2 which is encoded by the nucleotide sequence from nucleotide 2302 to nucleotide 2376 of SEQ ID NO: 1. This transmembrane domain could be mediating membrane attachment of the ACE-2 to a cell membrane.

A BLAST search (Altschul et al. (1990) J. Mol. Biol. 215:403) of the nucleic  
30 acid and the amino acid sequences of ACE-2 revealed that ACE-2 has significant homology to portions of angiotensin converting enzymes. An alignment of the amino acid sequence of human ACE-2 having SEQ ID NO: 2 with human testicular ACE (SEQ ID NO: 4; GenBank Accession No. P22966), murine testicular ACE (SEQ ID NO: 5; GenBank Accession No. P22967), rabbit testicular ACE (SEQ ID NO: 6; GenBank Accession No. P22968), human endothelial ACE (SEQ ID NO: 7; GenBank Accession No. P12821; U.S.  
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Patent No. 5,539,045 by Soubrier et al.; and described in Soubrier et al. (1988) Proc. Natl. Acad. Sci. USA 85:9386), murine endothelial ACE (SEQ ID NO: 8; GenBank Accession No. P09470), rat endothelial ACE (SEQ ID NO: 9; GenBank Accession No. P47820) and rabbit endothelial ACE (SEQ ID NO: 10; GenBank Accession No. P12822) is shown in Figure 2. The alignment was performed using CLUSTAL W (1.7). This alignment shows that the zinc binding domain is conserved among all ACE proteins. Other regions of the ACE-2 disclosed herein, such as the N-terminal region, are significantly different from the other ACE proteins. As further described herein, the previously described ACE proteins exist in two forms, a long form, referred to as endothelial ACE, and a short form, referred to as testicular ACE. Endothelial ACE is expressed in various tissues, whereas testicular ACE is expressed predominantly in developing sperm cells in the testis. Both of these forms derive from the same gene by alternative transcription initiation. The testicular ACE is in fact encoded by a mRNA whose transcription initiation site is located in an intron of the ACE gene. Thus, except for the first 67 N-terminal residues (including the 31 amino acid long signal peptide), human testis ACE is identical to the second half of the human endothelial ACE (Ehlers et al. (1989) Proc. Natl. Acad. Sci. USA 86:7741). Furthermore, the previously cloned ACE proteins from different species have a significant homology to each other, which is stronger than the homology of ACE-2 to any of the ACE proteins. Thus, the ACE-2 protein is encoded by a newly identified gene, having sequence similarities with some regions of the genes encoding the previously described ACE proteins, such as in the zinc binding domain.

The amino acid sequence comparison indicates that ACE-2 having SEQ ID NO: 2 has the highest overall similarity to the human testicular ACE and that it is 42.9% identical and 62% similar to the amino acid sequence of human testicular ACE. The cDNAs encoding human testicular ACE and ACE-2 (SEQ ID NO: 1) have an overall identity of 50.8%.

Figure 3 shows an amino acid alignment of the ACE-2 protein having SEQ ID NO: 2 and ACE proteins from *Drosophila Melanogaster* (SEQ ID NO: 11; GenBank Accession No. Q10714) and *C. Elegans* (SEQ ID NO: 12; GenBank Accession No. U56966), as well as human testicular and endothelial ACE. This alignment indicates that ACE-2 has a certain degree of homology with the *Drosophila* ACE protein, in particular in the zinc binding domain. However, ACE-2 does not have any significant homology with the *C. elegans* ACE protein.

Thus, based on the results of the BLAST analysis, ACE-2 is likely to be a second member of a novel family of angiotensin converting enzymes.

The BLAST analysis of GenBank with ACE-2 nucleic acid also indicated homologies of portions of human ACE-2 with the following ESTs:

**Table II**  
**Homologies of hu ACE-2 cDNA Sequence with EST Sequences**

Accession No.	Species	Nucleotides of SEQ ID NO:1	% Identity
AA397955	human	2759-3202 (3' UTR)	99%
AA420969	human	2936-3368 (3' UTR)	99%
AA162058	mouse	457-1012 (coding region)	87%
AA416585	human	2985-3368 (3' UTR)	100%
AA421125	human	2987-3287 (3' UTR)	100%
AA072298	mouse	1485-1742 (coding region)	83%

Among these ESTs, only AA162058 was annotated in GenBank as being homologous to ACE. A 208 bp fragment of a gene having Accession No. Q04027, annotated as human angiotensin converting enzyme, is 61% identical to nucleotides 1144-1353 of human ACE-2 cDNA having SEQ ID NO: 1.

#### 5.2. Tissue Distribution of ACE-2

A 167 bp fragment of human ACE-2 cDNA, corresponding to nucleotides 1152-1318 of SEQ ID NO: 1, was labeled with <sup>32</sup>P using the Multiprime Labeling System from Amersham and hybridized at 10<sup>6</sup> cpm/ml to Multiple Tissue Northern blots from Clontech overnight at 65 °C in Nylon Wash. The blots were then washed three times for 30 minutes at 65 °C in 0.5 x Nylon Wash. The results indicated that the ACE-2 probe hybridized to a mRNA of about 4 kb in kidney and testis. Thus, the size of the ACE-2 cDNA correlates with the size of the message RNA to which it hybridizes, indicating that the ACE-2 cDNA sequence is likely to be complete. The results also suggest that ACE-2 has a similar tissue distribution to that of the previously identified ACE. Thus, ACE-2 does not seem to be expressed solely in testis, as is characteristic of testicular ACE, but has a tissue distribution that is more like that of endothelial ACE, which is produced by many somatic tissues.

mRNA level of ACE-2 was also determined in a normal heart and compared to that of a heart of a congestive heart failure patient. For this, a 167 bp probe,

corresponding to nucleotides 1152-1318 of SEQ ID NO: 1, from the ACE homolog was hybridized to heart RNA from a normal and from 3 congestive heart failure patients, by Northern blot hybridization. 10 µg RNA was run per lane on a 1.2 % MOPS/formaldehyde gel, which was then transferred to Hybond N (Amersham) in 10 x SSC and crosslinked in a Stratalinker (Stratagene). The blot was hybridized overnight at 65 °C with 1 x 10<sup>6</sup> cpm/ml of Nylon wash. The ACE-2 probe hybridizes to the normal heart sample and to two of the three congestive heart failure samples.

### 5.3. Expression of Recombinant ACE-2 in COS Cells

This example describes a method for producing recombinant full length human ACE-2 in a mammalian expression system.

An expression construct containing a nucleic acid encoding a full length human ACE-2 protein, or a soluble ACE-2 protein which is devoid of the signal sequence and the transmembrane domain can be constructed as follows. A nucleic acid encoding the full length human ACE-2 protein or the soluble ACE-2 protein is obtained by reverse transcription (RT-PCR) of mRNA extracted from human cells expressing ACE-2, e.g., human kidney cells using PCR primers based on the sequence set forth in SEQ ID NO: 1. The PCR primers further contain appropriate restriction sites for introduction into the expression plasmid. The amplified nucleic acid is then inserted in a eukaryotic expression plasmid such as pcDNA1/Amp (InVitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gens, 3) *E. coli* replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA fragment encoding the full length human ACE-2 and a HA or myc tag fused in frame to its 3' end is then cloned into the polylinker region of the. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37, 767). The infusion of HA tag to ACE-2 allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

For expression of the recombinant ACE-2, COS cells are transfected with the expression vector by DEAE-DEXTRAN method. (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the ACE-2 -HA protein can be detected by radiolabelling and immunoprecipitation with an anti-HA antibody. (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). For this, transfected cells are labelled with <sup>35</sup>S-cysteine two days post transfection. The cells, or alternatively the

culture media (e.g., for the soluble ACE-2) is then collected and the ACE-2 protein immunoprecipitated with an HA specific monoclonal antibody. To determine whether full length ACE-2 is a membrane protein, and/or a secreted protein, the cells transfected with a vector encoding the full length ACE-2 protein can be lysed with detergent (RIPA buffer (150 mM NaCl 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM Tris, pH 7.5). (Wilson, I. et al., Id. 37:767 (1984)). Proteins precipitated can then be analyzed on SDS-PAGE gel. Thus, the presence of ACE-2 in the cell will be indicative that the full length ACE-2 can be membrane bound and the presence of ACE-2 in the supernatant will be indicative that the protein can also be in a soluble form, whether produced as a secreted protein or released by leakage from the cell.

#### 5.4 Angiotensin I is a substrate of ACE-2

This Example demonstrates that angiotensin I is a substrate of ACE-2 and that ACE-2 cleaves the C-terminal amino acid from angiotensin I and thus forms Ang. (1-9) having the amino acid sequence DRVYIHPFH (SEQ ID NO: 16).

The effect of ACE-2 on angiotensin I was tested as follows, and compared with the effect of ACE on these peptides. Incubation mixtures containing buffer (10 mM Tris, pH 7), ACE or ACE-2, and/or the ACE inhibitor Lisinopril were set up in six microtubes as set forth in Table III. 5 $\mu$ L of angiotensin I (DRVYIHPFHL; SEQ ID NO: 15) (15 pmol/ $\mu$ L obtained from Sigma-Aldrich Corp. (St. Louis, MO) was added to each microtube, following which the reaction mixtures were incubated for 30 minutes at 37°C.

ACE and ACE-2 polypeptides were produced in CHO cells as follows. Full length ACE-2 expression vector (pFLA-2) was generated by ligating an EcoRI/Afl III fragment of a 5' RACE clone (cchr002) and an Afl III/Not I fragment of the original library clone into the EcoRI/Not I sites of pcDNA3.1 (Invitrogen). An expression vector for secreted ACE-2 (pMD37) was generated by subcloning into pACE-2 a small PCR fragment which has a stop codon inserted after the serine immediately preceding the transmembrane domain. Testicular ACE cDNA was obtained from James Riordan and was subcloned into pcDNA3.1 for expression. For the transfections, CHO K1 cells, which are maintained in serum-free Ultra CHO medium (Biowhittaker) at 37 °C in a humidified 5% CO<sub>2</sub> incubator, were seeded at 1 x 10<sup>6</sup> cells/10 cm dish on day 0 and transfected with lipofectamine on day 1 as per manufacturers' instructions. Briefly, 10  $\mu$ g of DNA were combined with 40 $\mu$ l of lipofectamine in Opti-MEM medium and the mixture was incubated on the cells for 5-6 hours. Ultra CHO medium was then added to the cells and they were incubated overnight

at 37 °C. On day 2, the medium was changed and on day 4 the media were collected. For some experiments, the conditioned media were concentrated in Centriplus 30 concentrators (Amicon).

5

**Table III**

Content of enzymatic reactions with ACE enzymes

Microtube#	Buffer, Enzyme and Inhibitor Content
1	10 $\mu$ L of CHO cell lysate in buffer (negative control)
2	10 $\mu$ L of testicular ACE expressed in CHO cell lysate in buffer (positive control)
3	10 $\mu$ L of ACE-2 expressed in CHO cell lysate in buffer
4	10 $\mu$ L Endothelial ACE (0.33 mg/mL)(Sigma)
5	10 $\mu$ L of testicular ACE expressed in CHO cell lysate in buffer, 0.2 $\mu$ L of Lisinopril (500 pmol/ $\mu$ L)
6	10 $\mu$ L of ACE-2 expressed in CHO cell lysate in buffer, 0.2 $\mu$ L of Lisinopril (500 pmol/ $\mu$ L)

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After the enzymatic reaction, 1  $\mu$ L samples were removed from each reaction microtube and the enzymatic reaction was quenched by the addition of 1  $\mu$ L of a low-pH MALDI matrix compound, 10g/L  $\alpha$ -cyano-4 hydroxycinnamic acid ( $\alpha$ -CHCA) in a 1:1 mixture of acetonitrile and water (MALDI matrix solution). 1  $\mu$ L of each of the resulting quenched reaction mixtures was applied to the surface of a MALDI plate. The plate was then air-dried and inserted into the sample introduction port of a Voyager Elite Biospectrometry MALDI time-of-flight (TOF) mass spectrometer (PerSeptive Biosystems, Inc., Framingham, MA). The resulting signal was digitized at 1 GHz frequency and accumulated for 64 scans.

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The MALDI TOF MS mass spectrum of the angiotensin I used in these examples is shown in Figure 4A. The peak at  $m/z$  1296.68 corresponds to angiotensin I (DRVYIHPFHL; SEQ ID NO: 15). The peak at  $m/z$  1181.65 corresponds to angiotensin I lacking the N-terminal aspartic acid residue, i.e., angiotensin (2-10) (SEQ ID NO: 21), which results from cleavage by an aminopeptidase present in the angiotensin I solution.

Figure 4 B shows the MS spectrum of angiotensin I after having been incubated in the presence of a CHO cell lysate (= negative control; reaction 1 in Table III). The MS spectrum shows some conversion of angiotensin I to angiotensin fragment 1-7 (DRVYIHP, SEQ ID NO: 18,  $m/z$  899.26), which results from cleavage of angiotensin I by endopeptidase 24.11, which is present in the CHO cell lysate. Endopeptidase 24.11 cleaves angiotensin I between the proline and phenylalanine residues, thereby forming angiotensin (1-7).

Figure 4C is an MS spectrum of a reaction mixture containing angiotensin I after incubation with testicular ACE in a CHO cell lysate (positive control; reaction 2 in Table III). The spectrum shows total conversion of angiotensin I to angiotensin II by cleavage of the dipeptide histidine-leucine from angiotensin I, as evidenced by the absence of a peak at  $m/z$  1296.68, corresponding to angiotensin I, and the presence of a peak at  $m/z$  1046.79, corresponding to angiotensin II (DRVYIHPF; SEQ ID NO: 17)).

Figure 4D is an MS spectrum of a reaction mixture containing angiotensin I incubated with ACE-2 in a CHO cell lysate (reaction 3 in Table III). The presence of a peak at  $m/z$  1183.53, corresponding to angiotensin fragment 1-9 (DRVYIHPFHL; SEQ ID NO: 16), in the spectrum indicates that ACE-2 cleaves angiotensin I (DRVYIHPFHL) into angiotensin fragment 1-9 (DRVYIHPFH; SEQ ID NO: 16). Thus, ACE-2 does not have the identical enzymatic activity as testicular ACE or endothelial ACE, since testicular ACE and endothelial ACE convert angiotensin I to angiotensin II (DRVYIHPF, SEQ ID NO: 17,  $m/z$  1046.79). To confirm the identity of peak at  $m/z$  1183.53 as corresponding to angiotensin (1-9), the peptide was sequenced by electrospray tandem mass spectrometry (ESI-MS/MS). The results, which are shown in Figure 5 confirm that the peptide, resulting from the action of ACE-2 on angiotensin I is angiotensin (1-9) (DRVYIHPFH, SEQ ID NO: 16).

In another reaction, the effect of the ACE inhibitor Lisinopril was tested on ACE-2. In this reaction, angiotensin I was incubated with ACE-2 in a CHO cell lysate and Lisinopril (reaction 6 in Table III). The presence of a peak at  $m/z$  1183.54 in the spectrum (Figure 6B), which corresponds to angiotensin (1-9), and comparison of this spectrum with that resulting from incubation of angiotensin I with ACE-2 (Figure 4D), indicates that the ACE inhibitor Lisinopril has no effect on ACE-2 activity. However, as expected, Lisinopril inhibits testicular ACE enzymatic activity, as demonstrated by the absence of a peak at  $m/z$

1046.79, corresponding to angiotensin II, in the spectrum (Figure 6A) obtained from a reaction mixture in which angiotensin I was incubated with testicular ACE in a CHO lysate and Lisinopril (reaction 5 in Table III).

Thus, this Example demonstrates that ACE-2 cleaves angiotensin I into angiotensin (1-9) and that this enzymatic reaction is not inhibited by the ACE inhibitor Lisinopril.

#### 5.5 Kinetensin is a substrate of ACE-2

This Example demonstrates that kinetensin is a substrate of ACE-2 and that ACE-2 cleaves the C-terminal leucine residue from kinetensin.

Kinetensin (IARRHPYFL; SEQ ID NO: 23) obtained from Sigma-Aldrich Corp. (St. Louis, MO) was incubated at 15pmol/ $\mu$ L with ACE-2 produced in CHO cells as described above, and the reaction mixture was then subjected to mass spectrometry, also as described above. The results indicate that ACE-2 cleaves the C-terminal leucine residue from kinetensin, thus producing an 8-mer peptide having the amino acid sequence IARRHPYF (SEQ ID NO: 24).

#### 5.6 Effect of ACE-2 on other peptides

To determine whether ACE-2 is capable of cleaving peptides other than angiotensin I, ACE-2 produced in CHO cells, alone or with testicular ACE produced in CHO cells, was incubated as described above in 5  $\mu$ L reactions containing 15pmol/ $\mu$ L of each of the following peptides: angiotensin II (=angiotensin (1-8)), angiotensin (1-7), angiotensin (1-9), angiotensin (2-10), luteinizing hormone releasing hormone (LHRH), des-Gly<sup>10</sup>-LHRH Ethylamide, LHRH fragment 4-10, oxytocin, bradykinin, Arg<sup>8</sup>-Vasopressin, litorin, eledoisin, ranatensin, bombesin, neurotensin, renin substrate tetradecapeptide, and adrenocorticotrophic hormone fragment 18-39. These peptides were obtained from Aldrich Corp. (St. Louis, MO). MS spectrum of the reactions were obtained as described in the previous Example. The results showed that ACE-2 had no effect on any of these peptides, alone or in the presence of testicular ACE.

**Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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**CLAIMS**

1. An isolated nucleic acid comprising a nucleotide sequence which is at least about 70% identical to the entire nucleotide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3 or complement thereof.
2. An isolated polypeptide comprising an amino acid sequence having an amino acid identity of at least about 70% with the entire amino acid sequence set forth in SEQ ID NO: 2.
3. The isolated polypeptide of claim 2, which is a mammalian polypeptide.
4. The isolated polypeptide of claim 3, wherein the polypeptide is a human polypeptide.
5. The isolated polypeptide of claim 4, which is encoded by the nucleic acid having ATCC Designation No. 209510.
6. The isolated polypeptide of claim 4, which is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO: 1.
7. The isolated polypeptide of claim 6, which has the amino acid sequence set forth in SEQ ID NO: 2.
8. An isolated polypeptide comprising an amino acid sequence which is at least about 90% identical to at least about 15 consecutive amino acid residues of SEQ ID NO: 2.
9. The isolated polypeptide of claim 9, which has a bioactivity of an ACE-2 polypeptide.
10. The isolated polypeptide of claim 9, which binds a target peptide.
11. The isolated polypeptide of claim 10, which binds angiotensin I.
12. The isolated polypeptide of claim 11, which hydrolyzes angiotensin I into angiotensin (1-9).
13. The isolated polypeptide of claim 10, which binds kinetensin.
14. The isolated polypeptide of claim 13, which hydrolyzes kinetensin into kinetensin (1-8).

15. The isolated polypeptide of claim 8, which is encoded by a nucleic acid which hybridizes to a nucleic acid having the nucleotide sequence set forth in SEQ ID NO: 1 or complement thereof.
- 5 16. An isolated polypeptide comprising an amino acid sequence which is at least about 70% similar to at least about 50 consecutive amino acid residues of SEQ ID NO: 2 and which has a bioactivity of an ACE-2 polypeptide.
17. A method for producing a polypeptide of claim 2, comprising incubating a host cell including a nucleic acid encoding the polypeptide of claim 2 operably linked to a regulatory element, to thereby produce the polypeptide of claim 2.
- 10 18. The method of claim 17, wherein the host cell is *in vivo*.
19. A method for producing a polypeptide of claim 8, comprising incubating a host cell including a nucleic acid encoding the polypeptide of claim 8 operably linked to a regulatory element to thereby produce the polypeptide of claim 8.
- 15 20. A method for identifying an ACE-2 therapeutic, comprising contacting an ACE-2 polypeptide with a test compound and determining an ACE-2 bioactivity, such that a difference in the bioactivity of the ACE-2 polypeptide contacted with the test compound relative to an ACE-2 polypeptide that was not contacted with the test compound, indicates that the test compound is an ACE-2 therapeutic.
- 20 21. The method of claim 20, wherein the ACE-2 bioactivity includes binding of an ACE-2 polypeptide to a binding partner, and the method comprises
  - (i) combining an ACE-2 polypeptide and an ACE-2 binding partner, and a test compound under conditions wherein, but for the test compound, the ACE-2 polypeptide and ACE-2 binding partner form a complex; and
  - 25 (ii) detecting the formation of an ACE-2 polypeptide/ACE-2 binding partner complex, such that a difference in the formation of an ACE-2 polypeptide/ACE-2 binding partner complex in the presence of a test compound relative to the absence of the test compound indicates that the test compound is an ACE-2 therapeutic.
22. The method of claim 21 for identifying an ACE-2 antagonist, comprising

(i) combining an ACE-2 polypeptide and an ACE-2 binding partner, and a test compound under conditions wherein, but for the test compound, the ACE-2 polypeptide and ACE-2 binding partner form a complex; and

(ii) detecting the formation of an ACE-2 polypeptide/ACE-2 binding partner complex, such that a reduction in the formation of an ACE-2 polypeptide/ACE-2 binding partner complex in the presence of a test compound relative to the absence of the test compound indicatives that the test compound is an ACE-2 antagonist.

23. The method of claim 21, wherein the ACE-2 binding partner is a target peptide or analog thereof.

24. The method of claim 23, wherein the binding partner is angiotensin I or a portion thereof or an analog thereof sufficient for binding to an ACE-2 polypeptide.

25. The method of claim 23, wherein the binding partner is angiotensin I or a portion thereof or an analog thereof sufficient for binding to, and being hydrolyzed by, an ACE-2 polypeptide.

26. The method of claim 23, wherein the binding partner is kinetensin or a portion thereof or an analog thereof sufficient for binding to an ACE-2 polypeptide.

27. The method of claim 26, wherein the binding partner is kinetensin or a portion thereof or an analog thereof sufficient for binding to, and being hydrolyzed by, an ACE-2 polypeptide.

28. The method of claim 21, wherein the ACE-2 polypeptide comprises at least 15 amino acids having an amino acid sequence which is at least about 90% identical to an amino acid sequence set forth in SEQ ID NO: 2.

29. The method of claim 21, wherein the ACE-2 polypeptide is encoded by a nucleic acid which hybridizes to a nucleic acid having the nucleotide sequence set forth in SEQ ID NO: 1.

30. The method of claim 20, wherein the ACE-2 bioactivity includes cleavage of a target peptide by an ACE-2 polypeptide, and the method comprises

(i) combining into a reaction mixture an ACE-2 polypeptide and an ACE-2 target peptide or analog thereof, and a test compound under conditions

wherein, but for the test compound, the ACE-2 polypeptide cleaves one or more amino acids from the ACE-2 target peptide or analog thereof, thereby producing an ACE-2 target peptide conversion product; and

5 (ii) detecting in the reaction mixture the presence of at least one of the ACE-2 target peptide or analog thereof, the ACE-2 target peptide conversion product, and the one or more amino acids, such that a difference in the level of at least one of the ACE-2 target peptide or analog thereof, the ACE-2 target peptide conversion product, and the one or more amino acids in the reaction mixture containing the test compound relative to a reaction mixture that does not contain the  
10 test compound indicatives that the test compound is an ACE-2 therapeutic.

31. The method of claim 30, for identifying an ACE-2 antagonist, comprising

(i) combining into a reaction mixture an ACE-2 polypeptide and an ACE-2 target peptide or analog thereof, and a test compound under conditions wherein, but for the test compound, the ACE-2 polypeptide cleaves one or more  
15 amino acids from the ACE-2 target peptide or analog thereof, thereby producing an ACE-2 target peptide conversion product; and

(ii) detecting in the reaction mixture the presence of at least one of the ACE-2 target peptide or analog thereof, the ACE-2 target peptide conversion product, and the one or more amino acids, such that a lower level of at least one of  
20 the ACE-2 target peptide conversion product and the one or more amino acids produced, or a higher level of the ACE-2 target peptide or analog thereof, in the reaction mixture containing the test compound relative to a reaction mixture that does not contain the test compound indicatives that the test compound is an ACE-2 antagonist.

25 32. The method of claim 30, wherein the target peptide is angiotensin I or a portion thereof or an analog thereof sufficient for being cleaved by an ACE-2 polypeptide.

33. The method of claim 30, wherein the target peptide is kinetensin or a portion thereof or analog thereof sufficient for being cleaved by an ACE-2 polypeptide.

30 34. The method of claim 30, wherein the ACE-2 polypeptide comprises at least 15 amino acids having an amino acid sequence which is at least about 90% identical to an amino acid sequence set forth in SEQ ID NO: 2.

35. The method of claim 30, wherein the ACE-2 polypeptide is encoded by a nucleic acid which hybridizes to a nucleic acid having the nucleotide sequence set forth in SEQ ID NO: 1.
- 5 36. The method of claim 30, wherein detecting in the reaction mixture the presence of at least one of the ACE-2 target peptide or analog thereof, the ACE-2 target peptide conversion product, and the one or more amino acids comprises obtaining a mass spectrum of the reaction mixture or of a part thereof.
- 10 37. A method for modulating a bioactivity of an ACE-2 polypeptide, comprising contacting an ACE-2 polypeptide with a compound which has been identified according to the method of claim 30, such that the ACE-2 activity is modulated.
38. A method for treating or preventing the development of an abnormal blood pressure or disease or disorder associated therewith in a subject, comprising administering to the subject an effective amount of a pharmaceutical composition comprising an ACE-2 therapeutic, such that the disease or disorder in the subject is treated or prevented.
- 15 39. The method of claim 37, wherein the disease is selected from the group consisting of hypertension, congestive heart failure, chronic heart failure, acute heart failure, myocardial infarction, atherosclerosis, and renal failure.
40. The method of claim 37, further comprising administering to the subject an agonist of an ACE polypeptide.
- 20 41. A method for determining whether a subject has or is at risk of developing a disease or condition which is caused or contributed to by an aberrant ACE-2 activity, comprising measuring in the subject or in a sample obtained from the subject at least one ACE-2 activity, wherein a difference in the ACE-2 activity relative to the ACE-2 activity in a normal subject indicates that the subject is at risk of developing a disease caused by or contributed to by an aberrant ACE-2 activity.
- 25 42. A method for identifying a substrate of an ACE-2 polypeptide, comprising
- (i) contacting an ACE-2 polypeptide with a test compound in a reaction mixture under conditions in which the ACE-2 polypeptide is able to cleave a substrate; and
- (ii) determining the mass spectrum of the reaction mixture of step (a) or a part thereof,
- 30

1

**Abstract of the Invention**

**Angiotensin Converting Enzyme Homolog and Therapeutic and  
Diagnostic Uses Therefor**

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The present invention relates to the discovery of novel genes encoding an angiotensin converting enzyme, Angiotensin Converting Enzyme-2 (ACE-2). The invention provides therapeutics, prognostic and diagnostics methods for treating blood pressure related disorders as well as various types of allergic conditions, among others. Also disclosed are screening assays for identifying compounds for treating and preventing these conditions.

Figure 1 (A)

GAATTCGGCTTCCATCCTAATACGACTCACTATAGGGCTCGAGCGGCCCGCCGGGGCAGGTATCTTGGCTCACAGGGGA 79

M S S S S W L L L S L V A V T A A Q S 19  
CG ATG TCA AGC TCT TCC TGG CTC CTT CTC AGC CTT GTT GCT GTA ACT GCT GCT CAG TCC 138

T I E E Q A K T F L D K F N H E A E D L 39  
ACC ATT GAG GAA CAG GCC AAG ACA TTT TTG GAC AAG TTT AAC CAC GAA GCC GAA GAC CTG 198

F Y Q S S L A S W N Y N T N I T E E N V 59  
TTC TAT CAA AGT TCA CTT GCT TCT TGG AAT TAT AAC ACC AAT ATT ACT GAA GAG AAT GTC 258

Q N M N N A G D K W S A F L K E Q S T L 79  
CAA AAC ATG AAT AAT GCT GGG GAC AAA TGG TCT GCC TTT TTA AAG GAA CAG TCC ACA CTT 318

A Q M Y P L Q E I Q N L T V K L Q L Q A 99  
GCC CAA ATG TAT CCA CTA CAA GAA ATT CAG AAT CTC ACA GTC AAG CTT CAG CTG CAG GCT 378

L Q Q N G S S V L S E D K S K R L N T I 119  
CTT CAG CAA AAT GGG TCT TCA GTG CTC TCA GAA GAC AAG AGC AAA CGG TTG AAC ACA ATT 438

L N T M S T I Y S T G K V C N P D N P Q 139  
CTA AAT ACA ATG AGC ACC ATC TAC AGT ACT GGA AAA GTT TGT AAC CCA GAT AAT CCA CAA 498

E C L L L E P G L N E I M A N S L D Y N 159  
GAA TGC TTA TTA CTT GAA CCA GGT TTG AAT GAA ATA ATG GCA AAC AGT TTA GAC TAC AAT 558

E R L W A W E S W R S E V G K Q L R P L 179  
GAG AGG CTC TGG GCT TGG GAA AGC TGG AGA TCT GAG GTC GGC AAG CAG CTG AGG CCA TTA 618

Y E E Y V V L K N E M A R A N H Y E D Y 199  
TAT GAA GAG TAT GTG GTC TTG AAA AAT GAG ATG GCA AGA GCA AAT CAT TAT GAG GAC TAT 678

G D Y W R G D Y E V N G V D G Y D Y S R 219  
GGG GAT TAT TGG AGA GGA GAC TAT GAA GTA AAT GGG GTA GAT GGC TAT GAC TAC AGC CGC 738

G Q L I E D V E H T F E E I K P L Y E H 239  
GGC CAG TTG ATT GAA GAT GTG GAA CAT ACC TTT GAA GAG ATT AAA CCA TTA TAT GAA CAT 798

L H A Y V R A K L M N A Y P S Y I S P I 259  
CTT CAT GCC TAT GTG AGG GCA AAG TTG ATG AAT GCC TAT CCT TCC TAT ATC AGT CCA ATT 858

G C L P A H L L G D M W G R F W T N L Y 279  
GGA TGC CTC CCT GCT CAT TTG CTT GGT GAT ATG TGG GGT AGA TTT TGG ACA AAT CTG TAC 918

S L T V P F G Q K P N I D V T D A M V D 299  
TCT TTG ACA GTT CCC TTT GGA CAG AAA CCA AAC ATA GAT GTT ACT GAT GCA ATG GTG GAC 978

Q A W D A Q R I F K E A E K F F V S V G 319  
CAG GCC TGG GAT GCA CAG AGA ATA TTC AAG GAG GCC GAG AAG TTC TTT GTA TCT GTT GGT 1038

L P N M T Q G F W E N S M L T D P G N V 339  
CTT CCT AAT ATG ACT CAA GGA TTC TGG GAA AAT TCC ATG CTA ACG GAC CCA GGA AAT GTT 1098

Q K A V C H P T A W D L G K G D F R I L 359  
CAG AAA GCA GTC TGC CAT CCC ACA GCT TGG GAC CTG GGG AAG GGC GAC TTC AGG ATC CTT 1158

M C T K V T M D D F L T A H H E M G H I 379  
ATG TGC ACA AAG GTG ACA ATG GAC GAC TTC CTG ACA GCT CAT CAT GAG ATG GGG CAT ATC 1218 ZBD

Q Y D M A Y A A Q P F L L R N G A N E G 399  
CAG TAT GAT ATG GCA TAT GCT GCA CAA CCT TTT CTG CTA AGA AAT GGA GCT AAT GAA GGA 1278

F H E A V G E I M S L S A A T P K H L K 419  
TTC CAT GAA GCT GTT GGG GAA ATC ATG TCA CTT TCT GCA GCC ACA CCT AAG CAT TTA AAA 1338

S I G L L S P D F Q E D N E T E I N F L 439  
TCC ATT GGT CTT CTG TCA CCC GAT TTT CAA GAA GAC AAT GAA ACA GAA ATA AAC TTC CTG 1398

L K Q A L T I V G T L P F T Y M L E K W 459  
CTC AAA CAA GCA CTC ACG ATT GTT GGG ACT CTG CCA TTT ACT TAC ATG TTA GAG AAG TGG 1458

R W M V F K G E I P K D Q W M K K W W E 479  
AGG TGG ATG GTC TTT AAA GGG GAA ATT CCC AAA GAC CAG TGG ATG AAA AAG TGG TGG GAG 1518



Figure 1(B)

M K R E I V G V V E P V P H D E T Y C D	499
ATG AAG CGA GAG ATA GTT GGG GTG GTG GAA CCT GTG CCC CAT GAT GAA ACA TAC TGT GAC	1578
P A S L F H V S N D Y S F I R Y Y T R T	519
CCC GCA TCT CTG TTC CAT GTT TCT AAT GAT TAC TCA TTC ATT CGA TAT TAC ACA AGG ACC	1638
L Y Q F Q F Q E A L C Q A A K H E G F L	539
CTT TAC CAA TTC CAG TTT CAA GAA GCA CTT TGT CAA GCA GCT AAA CAT GAA GGC CCT CTG	1698
H K C D I S N S T E A G Q K L F N M L R	559
CAC AAA TGT GAC ATC TCA AAC TCT ACA GAA GCT GGA CAG AAA CTG TTC AAT ATG CTG AGG	1758
L G K S E P W T L A L E N V V G A K N M	579
CTT GGA AAA TCA GAA CCC TGG ACC CTA GCA TTG GAA AAT GTT GTA GGA GCA AAG AAC ATG	1818
N V R P L L N Y F E P L F T W L K D Q N	599
AAT GTA AGG CCA CTG CTC AAC TAC TTT GAG CCC TTA TTT ACC TGG CTG AAA GAC CAG AAC	1878
K N S F V G W S T D W S P Y A D Q S I K	619
AAG AAT TCT TTT GTG GGA TGG AGT ACC GAC TGG AGT CCA TAT GCA GAC CAA AGC ATC AAA	1938
V R I S L K S A L G D K A Y E W N D N E	639
GTG AGG ATA AGC CTA AAA TCA GCT CTT GGA GAT AAA GCA TAT GAA TGG AAC GAC AAT GAA	1998
M Y L F R S S V A Y A M R Q Y F L K V K	659
ATG TAC CTG TTC CGA TCA TCT GTT GCA TAT GCT ATG AGG CAG TAC TTT TTA AAA GTA AAA	2058
N Q M I L F G E E D V R V A N L K P R I	679
AAT CAG ATG ATT CTT TTT GGG GAG GAG GAT GTG CGA GTG GCT AAT TTG AAA CCA AGA ATC	2118
S F N F F V T A P K N V S D I I F R T E	699
TCC TTT AAT TTC TTT GTC ACT GCA CCT AAA AAT GTG TCT GAT ATC ATT CCT AGA ACT GAA	2178
V E K A I R M S R S R I N D A F R L N D	719
GTT GAA AAG GCC ATC AGG ATG TCC CGG AGC CGT ATC AAT GAT GCT TTC CGT CTG AAT GAC	2238
N S L E F L G I Q P T L G P P N Q P F V	739
AAC AGC CTA GAG TTT CTG GGG ATA CAG CCA ACA CTT GGA CCT CCT AAC CAG CCC CCT GTT	2298
S I W L I V F G V V M G V I V V G I V I	759
TCC ATA TGG CTG ATT GTT TTT GGA GTT GTG ATG GGA GTG ATA GTG GTT GGC ATT GTC ATC	2358
L I F T G I R D R K K K N K A R S G E N	779
CTG ATC TTC ACT GGG ATC AGA GAT CGG AAG AAG AAA AAT AAA GCA AGA AGT GGA GAA AAT	2418
P Y A S I D I S K G E N N P G F Q N T D	799
CCT TAT GCC TCC ATC GAT ATT AGC AAA GGA GAA AAT AAT CCA GGA TTC CAA AAC ACT GAT	2478
D V Q T S F *	806
GAT GTT CAG ACC TCC TTT TAG	2499
AAAAATCTATGTTTTCTCTTGAGGTGATTTTGTGTATGTAAATGTTAATTTTCATGGTATAGAAAATATAAGATGAT	2578
AAAGATATCATTAATGTCAAACTATGACTCTGTTTCAGAAAAAATGTCCAAAGACAACATGGCCAAAGGAGAGAGC	2657
ATCTTCATTGACATTGCTTTCAGTATTTATTTCTGTCTCTGGATTGACTTCTGTTCTGTTTCTTAATAAGGATTTTGT	2736
ATTAGAGTATATTAGGGAAAGTGTGTATTTGGTCTCACAGGCTGTTTCAGGGATAATCTAAATGTAATGTCTGTTGAAT	2815
TTCTGAAGTTGAAAAACAAGGATATATCATTTGGAGCAAGTGTGGATCTTGATGGAATATGGATGGATCACTTGTAAAGG	2894
ACAGTGCCTGGGAAGTGGTGTAGCTGCAAGGATTGAGAATGGCATGCAATAGCTCACTTTTCATTTAATCCATTGTCAAG	2973
GATGACATGCTTTCTTCACAGTAACTCAGTTCAAGTACTATGGTGATTTGCCTACAGTGATGTTTGGAAATCGATCATGC	3052
TTTCTTCAAGGTGACAGGTCTAAAGAGAGAAGAATCCAGGGAACAGGTAGAGGACATTGCTTTTTCACCTCCAAGGTGC	3131
TTGATCAACATCTCCCTGACAACACAAAAGTACAGCCAGGGGCTCCGTGAAGTCCAGAGCATGCCTGATAGAACTC	3210
ATTTCTACTGTTCTCTAACTGTGGAGTGAATGGAAATTCCTCACTGTATGTTTACCCCTCTGAAGTGGGTACCCAGTCTCT	3289
TAAATCTTTTGTATTTGCTCACAGTGTTTGAGCAGTGCTGAGCACAAGCAGACACTCAATAAATGCTAGATTTACACA	3368
CTCAAAAAAAAAAAAAAGGGCGGCCGC	3396

TMD

Figure 2 (A)

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ace-2 -----
hu-ACET -----
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rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
MGAASGRRGPGLLPLP-----LLLLL-PPQFALALDPGLQFGNFSADZAGAQLFAQSYN
MGAASGQGRWPLSPPLMLSLLVLLLQP-SPAPALDPGLQPGNFSFDEAGAQLFAESYN
MGAASGQGRWPLSPPLMLSLLLLLLLPPSPAPALDPGLQPGNFSADZAGAQLFADSYN
MGAAPGRRGFRLLRPPPLP-LLLLLLRPPFAALTLDPLGLPGDFAADEAGARLFASSYN

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rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
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SSAEVVMFQSTVASWAHDTNITEENARRQEEAALVSQEFPAEVMGKKAKELYESIWNQFDT
SSAEVVMFQSTAAWAHDTNITEENARRQEEAALINQEFPAEVMGKKAKELYESIWNQFDT
SSAEQVLFIRSTAASWAHDTNITAENARRQEEAALLSQEFPAEWGRRLRSSMTRCGRTSFT

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ace-2 -----
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rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
FQLRRIIGAVRTLGSANLPLAKRQQYNALLSNMSRIYSTAKVCLPNKTATCWSLDPDLTN
SKLRRIIGSIRT LGPANLPLAQKQQYNALLSNMSRIYSTGKVCFFPKTATCWSLDPDLTN
QKLRIIGSVQTLGPANLPLTQRLQYNALLSNMSRIYSTGKVCFFPKTATCWSLDPDLTN
QSCAGSSGLCAFWECCQPAF-GQAAADNSLLSNMSQIYSTGRSASPTLPAAWSLDFDLNN

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rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
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ILASSRSYAKLLFAWEGWHDVAGIPLKPLYQDFTALSNEAYRQDDFDTGAYWRSWYSEF
ILASSRNYAKVLFWEGWHDVAGIPLRPLYQDFTALSNEAYRQDGFDTGAYWRSWYSEF
ILASSRSYAMLLFAWEGWHNAVGIPLKPLYQEFALTALSNEAYRQDGFDTGAYWRSWYDSE

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rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
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SFEESLEHIYHQLEPLYLNLHAYVRRALHRRYGDKYVNLRGPIPAHLGDMWAQSWENIY
SFEESLEHLYHQLEPLYLNLHAFVRRALHRRYGDKYINLRGPIPAHLGDMWAQSWENIY
TFEEDLERIYHQLEPLYLNLHAYVRRVLRHRRYGDRYINLRGPIPAHLGDMWAQSWESIY

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ace-2 -----
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mu-ACET -----
rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
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DMVVPFPDKPNLDVTSTMVQKGNATHMFRVSEEFFTSLELSMPPEFWAESMLEKPTDG
DMVVPFPDKPNLDVTSTMVQKGNATHMFRVAEEFFTSLELSMPPEFWAESMLEKPADG
DMVVPFPDKPNLDVTSTMVQKGNATHMFRVAEEFFTSLELSMPPEFWAESMLEKPEDG

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ace-2 -----
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rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
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REVVCASAWDFYNRKDFRIKQCTRVMTQELATVHHMGHVQYYLQYKDLVSLRRGANP
REVVCASAWDFYNRKDFRIKQCTRVMTDQLSTVHHMGHVQYYLQYKDLVSLRRGANP
REVVCASAWDFYNRKDFRIKQCTQVTMDQLSTVHHMGHVQYYLQYKQDQVSLRR-ANP

```

960660" 960660"

Figure 2 (B)

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ace-2 -----
hu-ACET -----
mu-ACET -----
rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
GFHEAIGDVLALSVSTFEHLHKIGLLDRVTNDTESDINYLLKMALEKIAFLPFGYLVQW
GFHEAIGDVLALSVSTFAHLHKIGLLDHVTNDIESDINYLLKMALEKIAFLPFGYLVQW
GFHEAIGDVLALSVSTFAHLHKIGLLDRVANDIESDINYLLKMALEKIAFLPFGYLVQW
GFHEAIGDVLALSVSTFAHLHKIGLLDHVTNDTESDINYLLKMALEKIAFLPFGYLVQW

ace-2 -----
hu-ACET -----
mu-ACET -----
rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
RWGVFSGRTPPSRYNFDWWYLRTKYQGICPPVTRNETHFDAGAKFHVPNVTPYIRYFVSF
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RWGVFSGRTPPSRYNFDWWYLRTKYQGICPPVARNETHFDAGAKFHIFSVTPYIRYFVSF
RWGVFSGRTPPSRYNFDWWYLRTKYQGICPPVARNETHFDAGAKFHIFSVTPYIRYFVSF

ace-2 -----
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mu-ACET -----
rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
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-----MGQGWATFGLPSFLFLL---LC
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VLQFQFHEALCKEAGYEGPLHQCDIYRSTKAGAKLRKVLQAGCSRFPWQEVKDMVGLDGL
VLQFQFHEALCKEAGHQGFLHQCDIYQSTQAGAKLKQVLQAGCSRFPWQEVKDLVGSGL
VLQFQFHEALCKEAGHQGFLHQCDIYQSTKAGAKLQVVLQAGCSRFPWQEVKDLVGSGL
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ace-2 -----
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rb-ACET -----
hu-ACE
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rat-ACE
rb-ACE
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CGHLLVLSQVATDHVTANQGITTQATTSQTTHQATI DQTTQI - PNLETDEAKADREV
VPSRVAARRVTVNQGTTSQATTSSKATTSIRATTHQTTAHQTTQS - ENLVTEAERSKEV
DAQPLLKYFQPVQWLQEQNQNGEVLGWPEYQWHPFLPDNYPEG - IDLVTEAERSKEV
DAKALLEYFQPVSQWLEEQNQNGEVLGWPEYQWRPFLPDNYPEG - IDLETDEAKADREV
DASALMEYFQPVSQWLEEQNQNGEVLGWPEYQWRPFLPDNYPEG - IDLETDEAKADREV
DAQPLLDYFQPVQWLQEQNQNGEVLGWPEYQWRPFLPDNYPEG - IDLVTEAERSKEV
* * * * *

ace-2 -----
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rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
DKFNHEAEDLFYQSSLASWNYNTNITEENVQNMNAGDKWSAFLKEQSTLAQMYELQEIQ
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EEYDRTAQVLLNEYAEANWNYNTNITIEGSKILLEKSTEVSNHTLKYGTQARKFDVSNFQ
EEYDRSFQAVWVNEYAEANWNYNTNITEASKILLQKNMQIANHTLTYGNWARRFDVSNFQ
EEYDRTSQVWVNEYAEANWNYNTNITETSKILLQKNMQIANHTLKYGTQARKFDVNQLQ
EEYDRTAQVLLNEYAEANWNYNTNITIEGSKILLEKSTEVSNHTLKYGTQARKFDVSNFQ
EEYDRSTAKVLWVNEYAEANWNYNTNITIEGSKILLQKNKEVSNHTLKYGTWAKTFDVSNFQ
EEYDRSFQAVWVNEYAEANWNYNTNITEASKILLQKNMQIANHTLTYGNWARRFDVSNFQ
::: : : : : * : : : : : : : : : : : : : : : : : : : : : : *

ace-2 -----
hu-ACET -----
mu-ACET -----
rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
NLTVKLQALQNGSSVLSSEDKSKRLNTILNTMSTIYSTGKVCNPDNPQECLLLEPGLN
NTTIKRIIKKVQDLERAAALPAQEEYNYKILLDMETTYSVATVCHPENG--SCLQLEPDLT
NSSIKRIIKKLQNLDRAVLPKELEEYNYQILLDMETTYSLSNICYTNG--TCMPLEPDLT
NATSKRIIKKVQDLQRAVLVPEKELEEYNYQILLDMETTYSVANVCRVDG--SCLQLEPDLT
NTTIKRIIKKVQDLERAAALPAQEEYNYKILLDMETTYSVATVCHPENG--SCLQLEPDLT
NSSIKRIIKKLQNLDRAVLPKELEEYNYQILLDMETTYSLSNICYTNG--TCMPLEPDLT
NSTIKRIIKKVQNDRAVLVPEKELEEYNYQILLDMETTYSVANVCRVDG--TCLSLPDLT
NATSKRIIKKVQDLQRAVLVPEKELEEYNYQILLDMETTYSVANVCRVDG--SCLQLEPDLT
* : * : : : : : * : : : : * * * * * : : * : * : * : * : *

ace-2 -----
hu-ACET -----
mu-ACET -----
rb-ACET -----
hu-ACE
mu-ACE
rat-ACE
rb-ACE
EIMANSLDYNERLWAWESWRSEVGKQLRPLYEEYVVLKNEMARANHYEDYGDYWRGDYEV
NVMATSRKYEDLLWAWEGWRDKAGRAILQFYPKYVELINQARLNGYVDAGDSWRSMYET
NMMATSRKYEELLWAWKSWRDKVGRAILPFFPKYVEFSNKIAKLNGYTDAGDSWRSLYES
NLMATSRKYEDLLWVWTSWRDKVGRAILPFFPKYVEFTNKAARLNGYVDAGDSWRSMYET
NVMATSRKYEDLLWAWEGWRDKAGRAILQFYPKYVELINQARLNGYVDAGDSWRSMYET
NMMATSRKYEELLWAWKSWRDKVGRAILPFFPKYVEFSNKIAKLNGYTDAGDSWRSLYES
NIMATSRKYEELLWVWTSWRDKVGRAILPFFPKYVEFSNKIAKLNGYSDAGDSWRSSYES
NLMATSRKYEDLLWVWTSWRDKVGRAILPFFPKYVEFTNKAARLNGYVDAGDSWRSMYET
::: * : : : * : : : : : : : : : : : : : : : : : : : : : *

```



Figure 2 (D)

ace-2	LGDKAYEWNNDNMYLFRSSVAYAMRQYFLKVKNQMILFGEEDVRVANLKPRI SFNFVTA
hu-ACET	LGWPEYNWTPN-----
mu-ACET	LGWPEYNWAPN-----
rb-ACET	LGWPEYTWTPN-----
hu-ACE	LGWPEYNWTPN-----
mu-ACE	LGWPEYNWAPN-----
rat-ACE	LGWPEYTWTPN-----
rb-ACE	LGWPEYTWTPN-----

\*\* \* \* \*

ace-2	PKNVSDIIPRTEVEKAIRMSRSRINDAFRLNDNSLEFLGIQFTLGPPNQPPVS
hu-ACET	-----SARSE-----GSLPDSGRVS-----FLGLDLD---AQQARVGQWLLFL
mu-ACET	-----TARAE-----GSTAESNRVN-----FLGLYLE---PQQARVGQWVLLFL
rb-ACET	-----SARSE-----GSLPDSGRVN-----FLGMNLD---AQQARVGQWVLLFL
hu-ACE	-----SARSE-----GSLPDSGRVS-----FLGLDLD---AQQARVGQWLLFL
mu-ACE	-----TARAE-----GSTAESNRVN-----FLGLYLE---PQQARVGQWVLLFL
rat-ACE	-----TARAE-----GSLPESSRVN-----FLGMYLE---PQQARVGQWVLLFL
rb-ACE	-----SARSE-----GSLPDSGRVN-----FLGMNLD---AQQARVGQWVLLFL

.\*: \* : : \* : : \* : : \* : : \*

ace-2	VVMGVIVVGIVILIFTGIRDRKKKNKARSGENPYASIDISKGENNPGFQNTDDVQTSFN
hu-ACET	GIALLVATLGLSQR LFSIR-HRSLHRHSHG-----PQFGSEVELRHS--
mu-ACET	GVALLVATVGLAHRLYNIRNHHSLRRPHRG-----PQFGSEVELRHS--
rb-ACET	GVALLLASLGLTQRLFSIR-YQSLRQPHHG-----PQFGSEVELRHS--
hu-ACE	GIALLVATLGLSQR LFSIR-HRSLHRHSHG-----PQFGSEVELRHS--
mu-ACE	GVALLVATVGLAHRLYNIRNHHSLRRPHRG-----PQFGSEVELRHS--
rat-ACE	GVALLVATVGLAHRLYNIHNNHSLRRPHRG-----PQFGSEVELRHS--
rb-ACE	GVALLLASLGLTQRLFSIR-YQSLRQPHHG-----PQFGSEVELRHS--

: : : : : \* : : : \* : : : \*

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Figure 1. The effect of the concentration of the *Agrobacterium* suspension on the transformation efficiency of *Agrobacterium* strains. The *Agrobacterium* strains were grown in YEA medium for 24 h at 28°C. The cell concentration was adjusted to 10<sup>8</sup> cells/ml. The cells were then mixed with the plant tissue and the transformation efficiency was determined. The results are shown as the mean ± SD of three independent experiments. The transformation efficiency was significantly different from the control (p < 0.05).

```

HUM_tACE      -----
HUM_ACE       GRTPPSRYNFDWWYLRTKYQGICPPVTRNETHFDAGAKFHVPNVTPYIRYFVSFVLQFQF
DROME_ACE     -----
ace-2         -----
CE ACE        GACLPVFTQEIKPKPELLPADEAPKDPEAVFSEGEPELFELDALDTPKNGSVVPVPEPEPKP

```

Figure 3 (B)

```

HUM_tACE      -----MGQGWATAGLPSLLFLLLCYGHPLL
HUM_ACE       HEALCKEAGYEGPLHQCDIYRSTKAGAKLRKVLQAGSSRPWQEVLKDMVGLDALDAQPLL
DROME_ACE     -----
ace-2         -----
CE_ACE        EPEPEPEPKPEPEPSPTPEPEPAIKFDNIESEDYGDVAETAASTQPDELNTEVIEQLVDT

HUM_tACE      VPSQEASQQVTVTHGTSSQATTSSQTTTHQATAHQTSAQSPNLVTDEAEASKFVEEYDRT
HUM_ACE       KYFQPVQTWLQEQNQNGEVLGWPEYQWHPPLPDNYPEG-IDLVTDEAEASKFVEEYDRT
DROME_ACE     -----MRLFLALLATLAVTQALVKEEIQAKEYLENLNKE
ace-2         -----MSSSSWLLLSLVAVTAAQSTIEEQAKTFLDKFNHE
CE_ACE        FLNTGSIASNKTNGKPVFANPVAQALVNSSNYWKTDLNLAQPGSIKDEEKLRSWLAGYEA
               . * : : :

HUM_tACE      SQVVWNEYAEANWNYNTNITTETSKILLQKNMQIANHTLKYGTQARKFDVNQLQNTTIKR
HUM_ACE       SQVVWNEYAEANWNYNTNITTETSKILLQKNMQIANHTLKYGTQARKFDVNQLQNTTIKR
DROME_ACE     LAKRTNVETEAAWAYGNSITDENKKNEISAEAKFMKEVASDTTKFQWRSYQSEDLKR
ace-2         AEDLFYQSSSLASWNYNTNITEENVQNMNAGDKWSAFLKEQSTLAQMYPLQEIQLNTVCL
CE_ACE        AIKVLREVALSGWRYFNDASPSLKLALDEAENVLTMFVRSTSMQAKQFDMASVTDEKVMR
               : : * * : : : : : : : : : : : : : :

HUM_tACE      IIKKVQDLERAALPAQELEEYKILLDMETTYSVATVCHPNGS---CLQLEPDLTNVMTAT
HUM_ACE       IIKKVQDLERAALPAQELEEYKILLDMETTYSVATVCHPNGS---CLQLEPDLTNVMTAT
DROME_ACE     QFKALTKLGYAALPEDDYAELLDTLSAMESNFAKVVKCDYKDKSTKCDLALDPEIEEVISK
ace-2         QLQALQQNGSSVLSEDKSKRLNTILNTMSTIYSTGKVCNPDNPQE-CLLLEPGLEIMAN
CE_ACE        QLGYSFEGMSALAPSRFADYSQAQAALNRDSKSTICDKDVPPP-CALQKIDMDSIFRN
               : : : : * . : : : : : : : : : : : : : :

HUM_tACE      SRKYEDLLWAWEGWRDKAGRAILQFYPKYVELINQAARLNGYVDAGDSWRSMYETP---
HUM_ACE       SRKYEDLLWAWEGWRDKAGRAILQFYPKYVELINQAARLNGYVDAGDSWRSMYETP---
DROME_ACE     SRDHEELAYYWFYDKAGTAVRSQFERYVELNKAALKNNFTSGAEAWLDEYEDD---
ace-2         SLDYNERLWAWESWRSEVGKQLRPLYEYVVLKNEMARANHYEDYGDYWRGDYEVNGVDG
CE_ACE        EKDASRLQHLWVSyvTAIAKSK-PSYNNIITISNEGAKLNGFANGAMWRSAFMSS--K
               . . . * : : : : : : : : : : : : : :

HUM_tACE      -----SLEQDLERLFOELQPLYLNLHAYVRRALHRRHYG-AQHINLEGPIPAHLLGNMWA
HUM_ACE       -----SLEQDLERLFOELQPLYLNLHAYVRRALHRRHYG-AQHINLEGPIPAHLLGNMWA
DROME_ACE     -----TFEQQLDIFADIRPLYQQIHGYVRFLRKHYG-DAVSETGPIPMHLLGNMWA
ace-2         YDYSRGLIEDVEHTFEEIKPLYEHLHAYVRAKLMNAY--PSYISPIGCLPAHLLGDMWG
CE_ACE        VHKAEDFLNKQIDKIYSTIQPFYQLLHAYMRRQLAGIYSNPVGLSKDGPIPAHLFGSLDG
               : : : : : : : : * * * * * : : : * * * * : :

HUM_tACE      QTWSNIYDLVVPFPSA--PSMDTTEAMLKQGWTPRRMFKEADDFTSGLLPPVPEFWNK
HUM_ACE       QTWSNIYDLVVPFPSA--PSMDTTEAMLKQGWTPRRMFKEADDFTSGLLPPVPEFWNK
DROME_ACE     QQWSEIADIVSPFPEK--PLVDVSAEMEKQAYTPLKMFQMGDDFTSMNLTCLPQDFWDK
ace-2         RFWTNLYSLTVPFGQK--PNIDVTDAVDQAWDAQRI FKEAEKFFVSVGLPNMTQGFWEW
CE_ACE        GDWSAHYEQTKEFEESSETPEAMLSAFNTQNYTTKMFVTAYRYFKSAGFPHLPKSYWTS
               * : . . * * . : : * : . : * . : * * : : . : *

HUM_tACE      SMLEKPTDGREVVCHAS-AWDFYNGKDFRIKQCTTVNLEDLVVAHHEMIGHIQYFMQYKDL
HUM_ACE       SMLEKPTDGREVVCHAS-AWDFYNGKDFRIKQCTTVNLEDLVVAHHEMIGHIQYFMQYKDL
DROME_ACE     SIEKPTDGRDLVCHAS-AWDFYLLDDVRIKQCTRVTDQLFTVHHELGHIQYFLQYQHQ
ace-2         SMLTDPGNVQKAVCHPT-AWDLGKG-DFRI L MCTKVMTDDFLTAHHEMIGHIQYDMAYAAQ
CE_ACE        SIFAR-VWSKDMICHPAALDMRAPNDFRVKACAQLGEPDFEQAHSLLVQTYQYLYKQD
               * : : : * : : * * : * : : * : : : * : : *

HUM_tACE      PVALREGANPGFHEAIGDVLALSVSSTPKHLHSLNLLSSEGGSD--EHDINFLMKMALDKI
HUM_ACE       PVALREGANPGFHEAIGDVLALSVSSTPKHLHSLNLLSSEGGSD--EHDINFLMKMALDKI
DROME_ACE     PFVYRTGANPGFHEAVGDVLSLSVSSTPKHLEKIGLLKDYVRDD--EARINQLFLTALDKI
ace-2         PFLLRNGANEGFHEAVGEIMSLSAATPKHLKSIIGLLSPDFQEDN-ETEINFLKQALTIV
CE_ACE        SLLFREQASPVITDAIANAF AHLSTNPHYLYSQKLVPSHLDIKDSVIINKLYKESLESF
               . . * * . : : : : : : : : * : . . * * * : *

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Figure 3 (C)

```

HUM_tACE      AFIPFSYLVDQWRWRVFDGSITKENYNQEWWSLRLKYQGLCPPVPRTQGDGDPGAKFHIP
HUM_ACE       AFIPFSYLVDQWRWRVFDGSITKENYNQEWWSLRLKYQGLCPPVPRTQGDGDPGAKFHIP
DROME_ACE     VFLPFAFTMDKYRWSLFRGEVDKANWNCAFWKLRDEYSGLIEPPVVRSEKDFDAPAKYHIS
ace-2         GTLPFTYMLEKWRWMVFKGEIPKDQWMKKWEMKREIVGVVEPVPHDETYCDPASLFHVS
CE_ACE        TKLPFTIAADNWRYELFDGTVPKNKLNDRWWEIRNKYEGVRSFPQPYNTSNLDALIHNSVS
               :*:  ::*: :* * : * :  :*.: : * : *  *  :.  :.

HUM_tACE      -SSVPYIRYFVSFIIQFQFHEALCQAAGHTG-----PLHKCDIYQSKEAGQRLATAMKL
HUM_ACE       -SSVPYIRYFVSFIIQFQFHEALCQAAGHTG-----PLHKCDIYQSKEAGQRLATAMKL
DROME_ACE     -ADVEYLRYLVSFIIQFQFYKSACIKAGQYDPDNVELPLDNCDIYGSARAGAAAFHNMLSM
ace-2         -NDYSFIRYYTRTLTYQFQFQALCQAQKHEG-----PLHKCDISNSTEAGQKLFNMLRL
CE_ACE        QVHSPATRTLISYVLKFQILKALCQRELFWL-----SEGCILSEDTT---EKLRETMKL
               *      :*:  ::*  :  :  :  :  :  :  :  :  :  :  :  :

HUM_tACE      GFSRPWPEAMQLITGQPNMSASAMLSYFKPLLDWLR TEN-----
HUM_ACE       GFSRPWPEAMQLITGQPNMSASAMLSYFKPLLDWLR TEN-----
DROME_ACE     GASKPWPDALEAFNGERIMSGKAIAEYFEPLRVWLEAEN-----
ace-2         GKSEPWTALALENVGAKNMNVRPLNLYFEPLFTWLKDQNKNSFVGWSTDWSPYADQSIKV
CE_ACE        GSSITWLKALEMISGKGELDAQPLLEYEPLINWLRNTN-----
               * * . * *:  . *  :.  . : .*:  * * . *

HUM_tACE      -ELHGEKLGWPQYNWTPNSAR-----
HUM_ACE       -ELHGEKLGWPQYNWTPNSAR-----
DROME_ACE     -IKNNVHIGWTTSNKCVSS-----
ace-2         RISLKSALGDKAYEWNDEMILFRSSVAYAMRQYFLKVKNQMILFGEEDVRVANLKPRIS
CE_ACE        -EIDQVVVGWDGEGTPTFVEEIPKTRQPGDGGNGLPSEDRVAFPGGE-----
               :*

HUM_tACE      -----SEGPLPDSGRVSFLGLDLDAQQARVG-----Q
HUM_ACE       -----SEGPLPDSGRVSFLGLDLDAQQARVG-----Q
DROME_ACE     -----
ace-2         FNFFVTAPKNVSDIIPRTEVEKAI RMSRSRINDAFRLNDNSLEFLGIQPTLGPPNQPPVS
CE_ACE        -----CVNGQECILDSHCNGTICVCNDGLYTL EIGNTFN---CVPGN

HUM_tACE      WLLLFLGIALLVATLGLSQRLFS-IRHR-----SLHRHSHGPQFGSEVE
HUM_ACE       WLLLFLGIALLVATLGLSQRLFS-IRHR-----SLHRHSHGPQFGSEVE
DROME_ACE     -----
ace-2         IWLIVGVVMGVIVVGIVILIFTGIRDRKKKNKARSGENPYASIDISKGENNPGFQNTDD
CE_ACE        PADSGFGDGKGLVIGLFENNEVTTPEPSAEPEP--TAKTTTKMPPRVRAATS PFSLYLTV

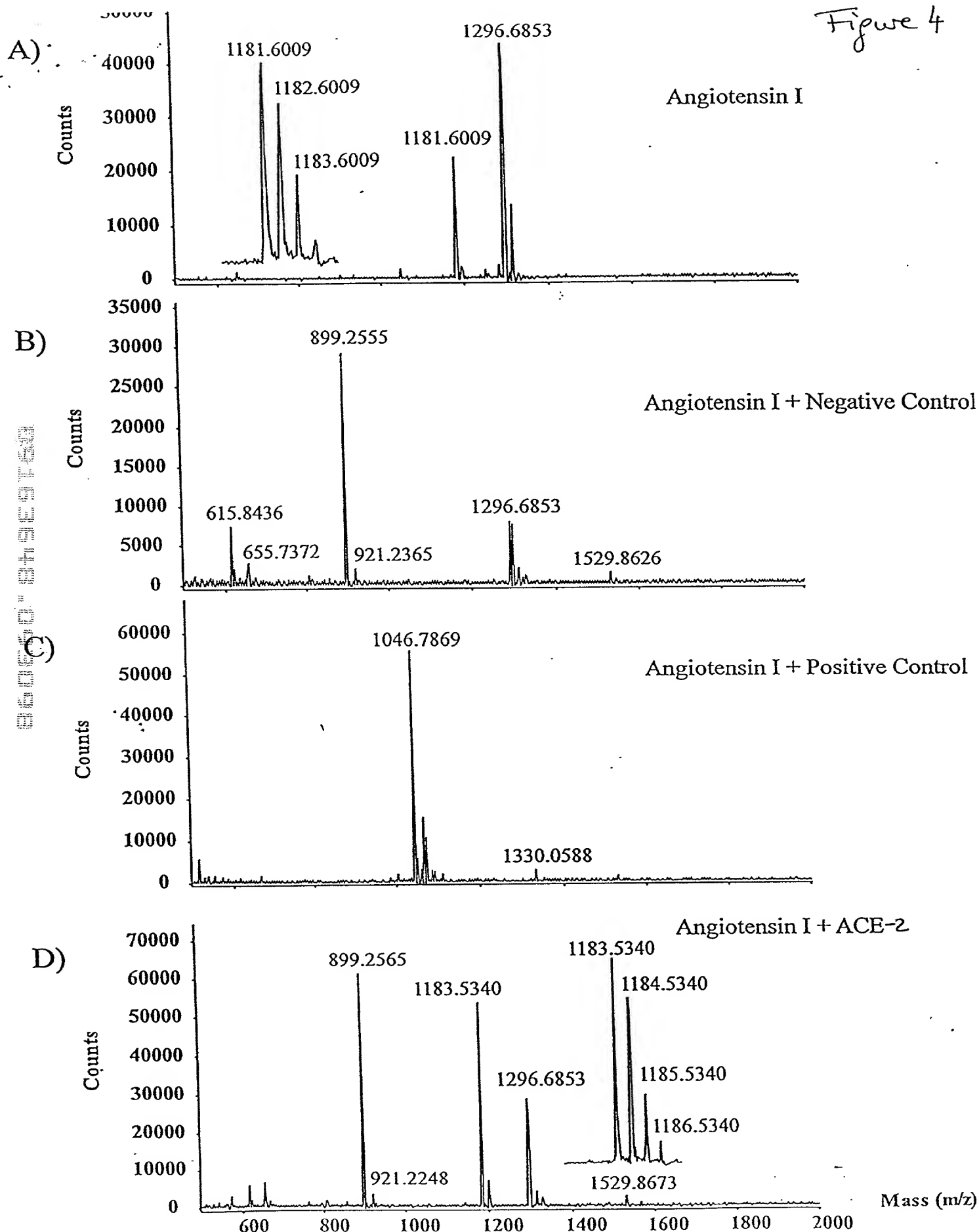
HUM_tACE      LRHS----
HUM_ACE       LRHS----
DROME_ACE     -----
ace-2         VQTSFN--
CE_ACE        LLIIYFAL

```

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Figure 4



860660" 849E3T60

1: TOF MSMS 395.40ES+  
310

acehomo2 175 (3.292) Cm (103:276)

Figure 5

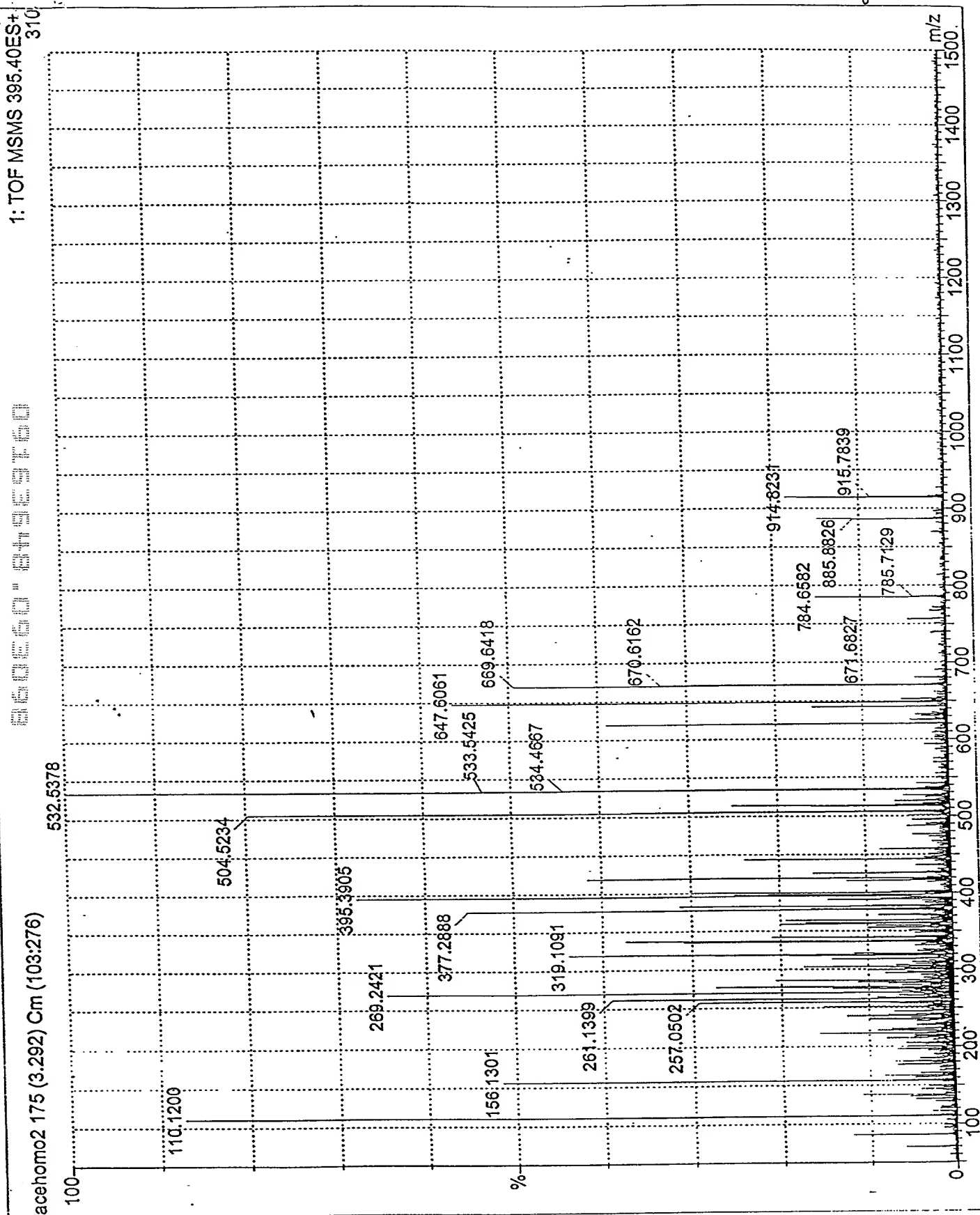
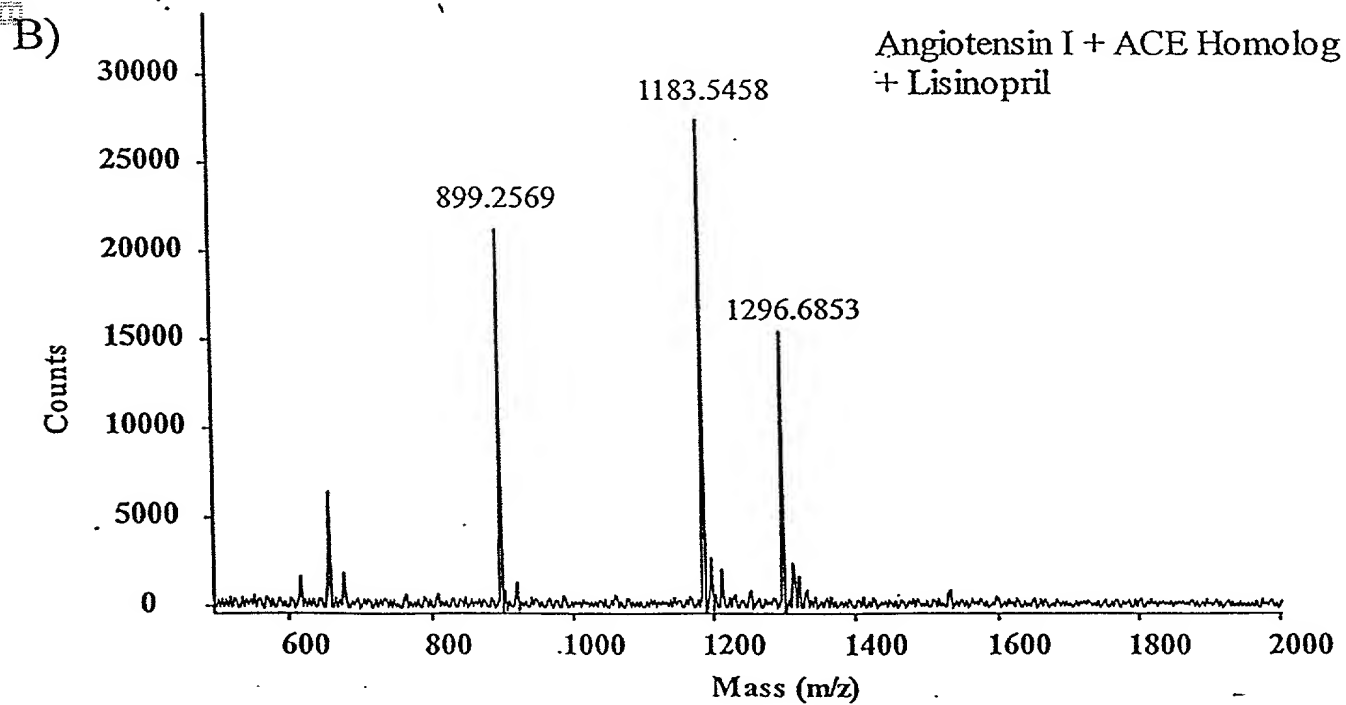
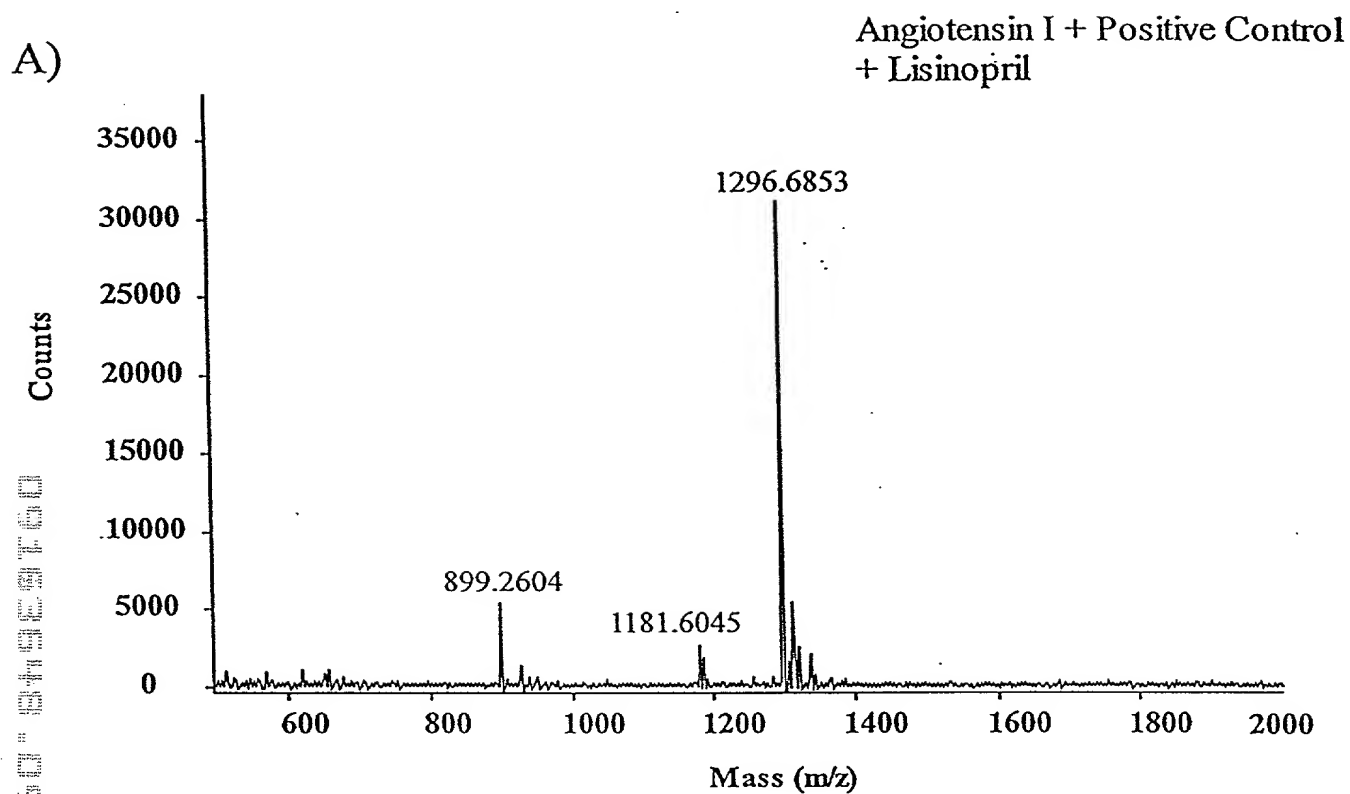


Figure 6



# Angiotensin I conversion to products

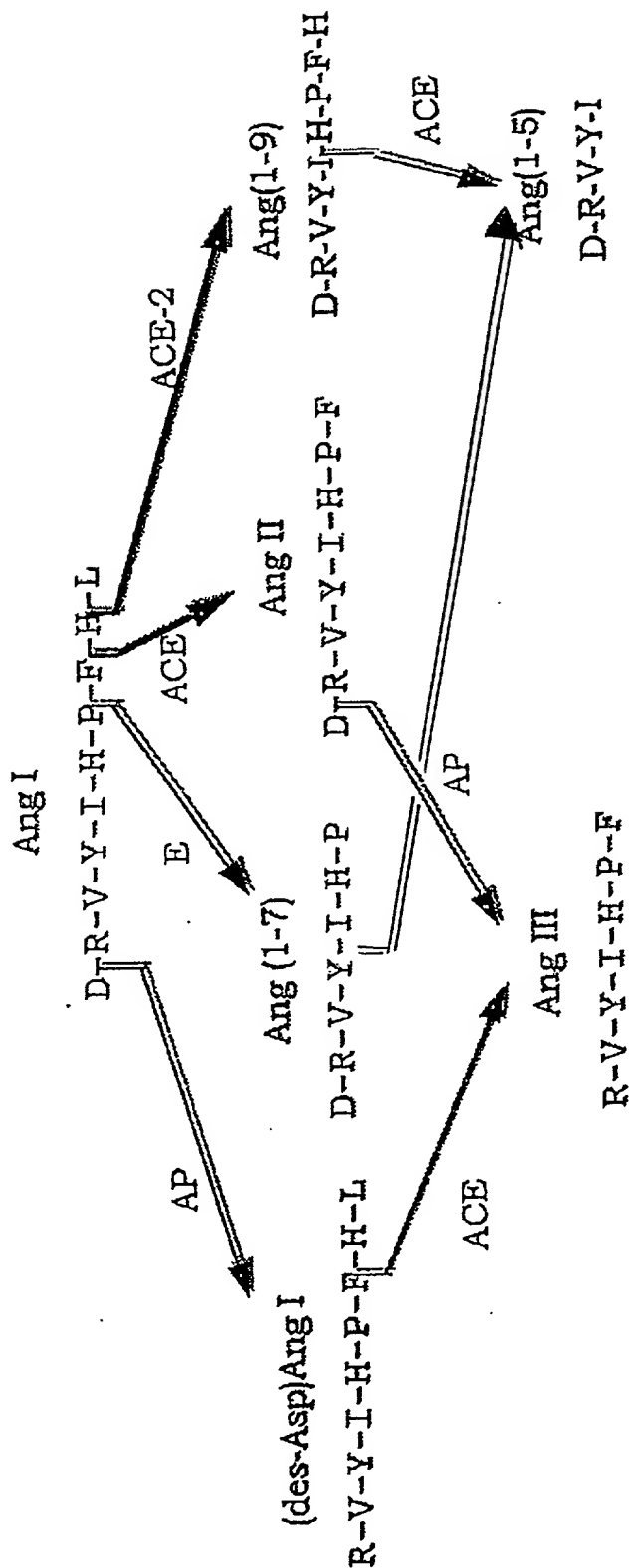


Figure 7.

Year	Total population		Urban population		Rural population		Total population		Urban population		Rural population	
	Population	Density	Population	Density	Population	Density	Population	Density	Population	Density	Population	Density
1950	1,000,000	100	500,000	50	500,000	50	1,000,000	100	500,000	50	500,000	50
1955	1,100,000	110	550,000	55	550,000	55	1,100,000	110	550,000	55	550,000	55
1960	1,200,000	120	600,000	60	600,000	60	1,200,000	120	600,000	60	600,000	60
1965	1,300,000	130	650,000	65	650,000	65	1,300,000	130	650,000	65	650,000	65
1970	1,400,000	140	700,000	70	700,000	70	1,400,000	140	700,000	70	700,000	70
1975	1,500,000	150	750,000	75	750,000	75	1,500,000	150	750,000	75	750,000	75
1980	1,600,000	160	800,000	80	800,000	80	1,600,000	160	800,000	80	800,000	80
1985	1,700,000	170	850,000	85	850,000	85	1,700,000	170	850,000	85	850,000	85
1990	1,800,000	180	900,000	90	900,000	90	1,800,000	180	900,000	90	900,000	90
1995	1,900,000	190	950,000	95	950,000	95	1,900,000	190	950,000	95	950,000	95
2000	2,000,000	200	1,000,000	100	1,000,000	100	2,000,000	200	1,000,000	100	1,000,000	100
2005	2,100,000	210	1,050,000	105	1,050,000	105	2,100,000	210	1,050,000	105	1,050,000	105
2010	2,200,000	220	1,100,000	110	1,100,000	110	2,200,000	220	1,100,000	110	1,100,000	110
2015	2,300,000	230	1,150,000	115	1,150,000	115	2,300,000	230	1,150,000	115	1,150,000	115
2020	2,400,000	240	1,200,000	120	1,200,000	120	2,400,000	240	1,200,000	120	1,200,000	120
2025	2,500,000	250	1,250,000	125	1,250,000	125	2,500,000	250	1,250,000	125	1,250,000	125
2030	2,600,000	260	1,300,000	130	1,300,000	130	2,600,000	260	1,300,000	130	1,300,000	130
2035	2,700,000	270	1,350,000	135	1,350,000	135	2,700,000	270	1,350,000	135	1,350,000	135
2040	2,800,000	280	1,400,000	140	1,400,000	140	2,800,000	280	1,400,000	140	1,400,000	140
2045	2,900,000	290	1,450,000	145	1,450,000	145	2,900,000	290	1,450,000	145	1,450,000	145
2050	3,000,000	300	1,500,000	150	1,500,000	150	3,000,000	300	1,500,000	150	1,500,000	150
2055	3,100,000	310	1,550,000	155	1,550,000	155	3,100,000	310	1,550,000	155	1,550,000	155
2060	3,200,000	320	1,600,000	160	1,600,000	160	3,200,000	320	1,600,000	160	1,600,000	160
2065	3,300,000	330	1,650,000	165	1,650,000	165	3,300,000	330	1,650,000	165	1,650,000	165
2070	3,400,000	340	1,700,000	170	1,700,000	170	3,400,000	340	1,700,000	170	1,700,000	170

Karen L. Bovard, Director  
Office of Enrollment and Discipline

## DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

***Angiotensin Converting Enzyme Homolog and Therapeutic and Diagnostic Uses Therefor***

the specification of which

(X) was filed on September 30, 1998, as United States Application Serial Number \_\_\_\_\_

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

This application in part discloses and claims subject matter disclosed in my earlier filed pending application,

Serial No. 08/989,299, Filed December 11, 1997,

and I hereby claim the benefit of said United States prior application under Title 35, United States Code, §120.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulation, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Claimed

( ) Yes ( ) No

(Number)

(Country)

(Day/Month/Year Filed)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States Provisional application(s) listed below.

(Application Number)

(Filing Date)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

I hereby appoint Mark Boshar, Reg. No. 35, 456; Robert Millman, Reg. No. 36, 217; Jean M. Silveri, Reg. No. 39,030; Theodore Allen, Reg. No. 41,578; Cynthia Kanik, Reg. No. 37,320; Beth E. Arnold, Reg. No. 35,430; Charles H. Cella, Reg. No. 38,099; Edward J. Kelly, Reg. No. 38,936; Donald W. Muirhead, Reg. No. 33,978; Isabelle M. Clauss, Reg. No. (see attached); Matthew P. Vincent, Reg. No. 36,709; and Anita Varma, Reg. No. 43,221 as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Post Office Address same as above

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